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N° bibliothèque : XXX

ROLE DU SILICIUM SUR LA TOLERANCE AU CUIVRE ET LA CROISSANCE DES BAMBOUS

THESE

pour obtenir le grade de

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ANNEE 2011

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INTRODUCTION

Face à la croissance démographique, la gestion des déchets et des eaux usées représente un enjeu technologique, économique et scientifique majeur des années à venir, aussi bien dans les pays du Nord que dans ceux du Sud. Il est indispensable de travailler à l'élaboration de solutions de traitement qui permettent une épuration optimale des eaux usées avant leur réutilisation ou leur rejet dans le milieu naturel. Afin de pallier les limites environnementales, économiques et sociétales des méthodes conventionnelles qui sont actuellement employées pour le traitement des sols pollués, telles que l'incinération et le confinement, les recherches s'orientent, depuis une quinzaine d'années, vers de nouvelles méthodes biologiques de traitement des sols basées sur l'utilisation des plantes. Ces méthodes, qui se regroupent sous le terme de phytoremédiation, apparaissent comme des voies alternatives, moins coûteuses, plus extensives et plus respectueuses des sols et de l'environnement (Jabeen et al. 2009; Keller 2005; Pilon-Smits 2005).

La société PHYTOREM® a développé une technologie d'épuration utilisant le procédé de phytoremédiation sur sol en place : le BAMBOU ASSAINISSEMENT®. Cette technologie est optimisée par le choix du bambou, plante ligneuse de la famille des *poaceae,* qui possède l'un des plus forts taux de croissance du règne végétal. Une forte production de biomasse, combinée à une forte capacité à absorber de l'eau et des éléments minéraux, permet au bambou d'être une plante adaptée aux techniques de phytoépuration (Arfi et al. 2009). Le BAMBOU ASSAINISSEMENT® est une technologie rustique et simple dans sa mise en œuvre et son exploitation. PHYTOREM® conçoit et gère des stations qui exploitent au maximum ces processus pour épurer plusieurs types d'effluents : eaux usées, effluents industriels et vinicoles, lixiviats, eaux pluviales, etc.

Les activités humaines liées aux évolutions industrielles des XIXe et XXe siècles ont déversé des quantités importantes d'éléments traces métalliques (ETM) dans les compartiments liquides, solides et gazeux de la terre, augmentant ainsi leur concentration dans le milieu naturel. Ces métaux sont non biodégradables et ont donc tendance à s'accumuler dans les déchets et les eaux

usées que nous produisons. Mais, jusqu'à présent, le comportement des bambous face à la présence de métaux n'a pas été évalué. Comme l'un des symptômes de la toxicité métallique est la réduction de la croissance des plantes, il convient de déterminer si l'efficience du BAMBOU ASSAINNISSEMENT® est affectée par la présence des ETM dans les effluents à traiter. Ainsi, il est nécessaire de comprendre le comportement conjoint des ETM et du bambou : comme leur capacité d'absorption, la localisation des éléments dans la plante et leur toxicité. Nous avons choisi le cuivre (Cu) comme ETM car c'est l'un des contaminants les plus abondants dans certains polluants qui peuvent être traités par le BAMBOU ASSAINNISSEMENT®, comme par exemple le lisier de porc (Legros et al. 2010). Le cuivre est également intéressant puisqu'il fait partie des oligoéléments : il est nécessaire pour le développement de la plante à faible dose mais induit une forte toxicité quand sa concentration cellulaire est trop élevée.

Le bambou possède la particularité d'absorber de fortes quantités de silicium, jusqu'à 40 % de matière sèche de SiO_2 dans les feuilles. Or de récentes études montrent que le silicium permet d'améliorer la croissance et la résistance des plantes soumises à des stress, notamment à une toxicité métallique (da Cunha and do Nascimento 2009; Gu et al. 2011). Ainsi il est donc intéressant, dans le but d'améliorer la technologie de phytoremédiation du bambou, d'étudier le rôle que peut avoir sur lui le silicium, en particulier lorsque celui-ci fait face à une toxicité métallique.

Les objectifs de ce travail de thèse sont les suivants :

1- Déterminer le rôle du silicium dans le bambou.
   - L'apport de silicium permet-il d'augmenter la biomasse du bambou ?
   - Les nombreuses espèces de bambous présentent-elles des différences d'accumulations de Si ?
   - L'amendement en silicium peut-il être envisagé pour le BAMBOU ASSAINNISSEMENT®?
2- Déterminer le comportement de Cu dans le bambou
   - Quelle est la répartition de Cu dans les différentes parties du bambou ?
   - A quelle concentration Cu devient toxique pour le bambou, quels sont les symptômes ?
   - Est-ce que Cu a un comportement différent en fonction de l'espèce de bambou ?
3- Déterminer si la présence de Si minimise la toxicité de Cu.

Cette étude approfondie des différents processus impliqués dans la toxicité de Cu et le rôle de Si dans le bambou a été réalisée à différentes échelles et en couplant plusieurs techniques analytiques.

Tout d'abord la répartition et la variabilité de Cu et Si ont été étudiées dans plusieurs espèces de bambous se développant dans un contexte pédoclimatique naturel. Comme aucune donnée de concentration de Cu et Zn n'existe dans la littérature, cette approche a donc permis d'établir des concentrations de références de Cu et Zn dans les bambous.

Ensuite, des expériences en culture hydroponique ont été réalisées afin de contrôler finement l'apport de Si et Cu aux bambous. Ces expériences ont permis de caractériser de manière « macroscopique » la réponse des bambous en fonction des apports de Si et à Cu. Parallèlement, nous avons étudié la spéciation de Cu au sein des différents organes du bambou. La spéciation (spectroscopie d'absorption des rayons X) associée à la localisation des éléments (microfluorescence X et microscopie électronique) sont deux caractérisations indispensables pour mieux appréhender le comportement de Cu dans le bambou.

Ce document s'articule en quatre parties principales :

- Dans le chapitre 1, sera présenté un état de l'art sur le comportement de Cu et de Si dans les plantes et le bambou en particulier.
- Le chapitre 2 décrit et discute la variabilité et la localisation de Si, Cu et Zn dans plusieurs espèces de bambous développées en conditions pédoclimatiques naturelles.
- Le chapitre 3 présente l'effet de Si et Cu sur des bambous cultivés en hydroponie.
- Le chapitre 4 traite de l'effet d'une toxicité au Cu chez le bambou et le rôle de Si, en culture hydroponique. Nous présenterons dans ce chapitre l'étude de la spéciation de Cu.

Ce projet de recherche a été mené dans le cadre d'une bourse CIFRE entre le CEREGE (Centre Européen de Recherche et d'Enseignement des Géosciences de l'Environnement) et la société PHYTOREM et d'un contrat de collaboration de recherche entre le CEREGE et PHYTOREM soutenu par la Direction Générale des Entreprises.

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CHAPITRE I

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CHAPITRE I

SYNTHESE BIBLIOGRAPHIQUE

Cette synthèse bibliographique est organisée en deux axes principaux dédiés au cuivre et au silicium dans les plantes.

Comme aucune donnée n'est encore publiée sur Cu dans le bambou, la première partie présente les connaissances actuelles sur la présence de Cu dans les plantes : ses caractéristiques, ses mécanismes de prélèvement, de transport et sa toxicité. Les approches et les outils utilisés pour caractériser le comportement de Cu dans les plantes sont nombreux et couvrent différentes échelles : de l'observation macroscopique des effets de Cu à des études de génomique. Comme nous avons utilisé la spectroscopie d'absorption des rayons X pour caractériser la spéciation du Cu dans les bambous, une présentation des principaux résultats de spéciation de Cu acquis grâce à cette méthode dans les plantes complète cette première partie.

La seconde partie détaille le comportement du silicium dans les plantes et dans le bambou : ses mécanismes de prélèvement, de transport, sa répartition dans les différents tissus du bambou, ses fonctions dans les plantes et son rôle face à une toxicité métallique.

1. LE CUIVRE DANS LES PLANTES

1.1. Le cuivre, oligo-élément

Le cuivre fait partie des métaux de transition, c'est un oligo-élément présent en proportion très faible dans les tissus biologiques mais indispensable à la vie. En 1930, Cu est reconnu élément trace essentiel pour les plantes (Alloway 1995). Certains éléments traces cationiques plurivalents comme Cu peuvent se présenter sous différents états d'oxydation ($Cu^{2+} + e^- \leftrightarrow Cu^+$) et jouent ainsi un rôle d'accepteur ou de donneur d'électrons, ce qui est très important dans les multiples systèmes enzymatiques mettant en jeu des réactions d'oxydoréduction. Le cuivre est également nécessaire à de nombreuses enzymes, comme activateur ou comme constituant spécifique des protéines (Marschner 1995). Dans le cas d'une croissance optimale, les concentrations en Cu dans les plantes atteignent des valeurs comprises entre 5 et 20 $mg.kg^{-1}$ de

matière sèche (MS) dans les parties aériennes (Marschner 1995). En dessous d'un seuil de 2-5 $mg.kg^{-1}$ MS dans la plante, sa croissance est sévèrement réduite et des symptômes de déficience peuvent se manifester (Figure I.1). Ce seuil peut varier suivant les espèces végétales et l'état de développement de la plante. A l'inverse, la teneur en Cu mesurée dans la plante peut atteindre une concentration critique à partir de laquelle apparaissent des symptômes de phytotoxicité (Marschner 1995).

Les concentrations de métaux libres ou chélates dans les solutions de sol sont généralement faibles, même si cela dépend des propriétés du sol (Marschner 1995). Que ce soit dans la solution ou dans la phase solide, Cu est majoritairement présent sous forme complexée. Les minéraux secondaires, comme les argiles et les oxyhydroxydes de Fe et de Mn et les matières organiques, sont les principaux constituants du sol qui contribuent à l'adsorption des éléments traces (Kabata-Pendias and Pendias 2001). En quantité moindre, Cu peut également être présent dans les réseaux cristallins d'autres minéraux secondaires comme les carbonates, les sulfures, les phosphates et certains oxydes (Das et al. 1995). La distribution de Cu parmi ces différents composants du sol influence sa mobilité et, ainsi, la quantité potentiellement disponible pour les plantes. La rétention de Cu par la phase solide du sol est fortement dépendante du pH. En effet, lorsque le pH du sol augmente, la charge nette de surface des phases adsorbantes devient de moins en moins positive (oxyhydroxydes Fe et Al) ou de plus en plus négative (oxyhydroxydes Mn et MOS), ce qui accroît leur affinité pour les cations métalliques (Alloway 1995).

Cependant le Cu biodisponible va également être influencé par les processus physiques, chimiques et biologiques qui ont lieu à l'interface sol-racine, dans la rhizosphère, tels qu'une modification du pH ou de la quantité de carbone organique dissous (Hinsinger et al. 2009).

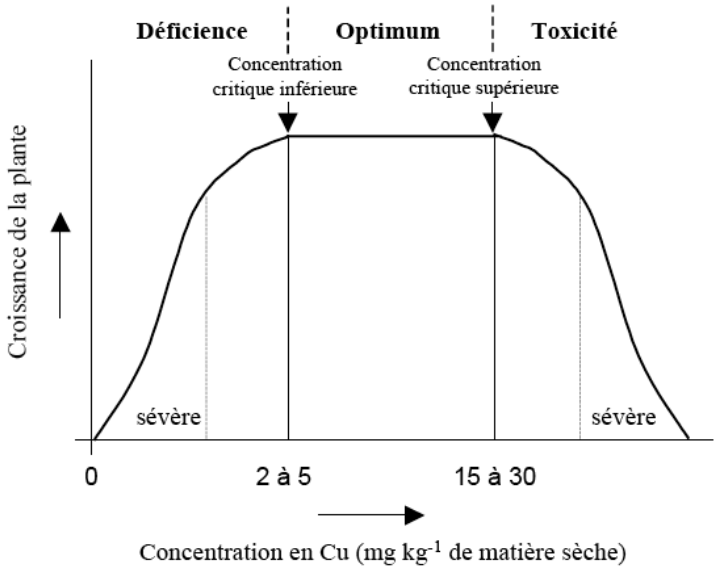


Figure I.1 **Courbe de croissance en réponse au statut nutritionnel de la plante, d'après Reuter and Robinson (1997)**

1.2. Mécanismes de prélèvement

Le cuivre pénètre dans la plante par son système racinaire. Le transfert radial des ions métalliques de la surface des racines jusqu'aux vaisseaux du xylème peut suivre deux voies (Figure I.2) :

- la *voie apoplasmique* en passant dans les espaces intercellulaires (en rouge). Le transfert des solutés de faible poids moléculaire (éléments traces complexés ou non, acides organiques, acides aminés, sucres) de la solution du sol vers l'espace pariétal (parois cellulaires et apoplasme ou espace intercellulaire) se fait passivement par diffusion (Marschner, 1995) avant d'être bloqué par la bande de Caspary dans l'endoderme.
- la *voie symplasmique* (en bleu) en traversant la membrane plasmique d'une cellule, généralement de la couche la plus externe du cortex, puis en passant de cellule à cellule par les plasmodesmes (les pores de la paroi cellulaire). Le transport des métaux dans le symplasme est pris en charge par différentes familles de transporteurs.

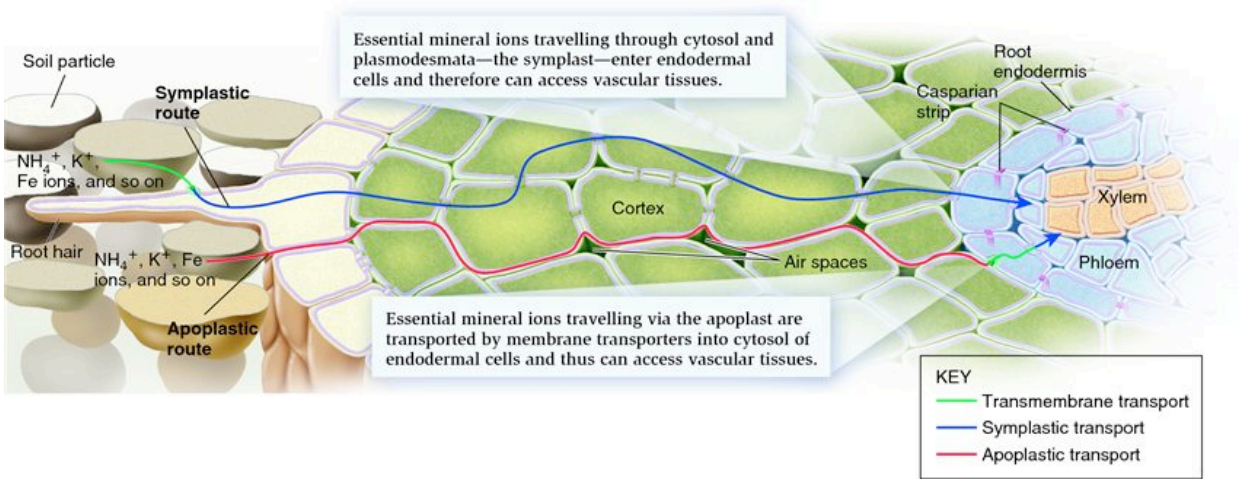


Figure I.2 **Transfert radial des minéraux dans une racine, de la solution de sol au xylème (image issue du site internet biologie-forums.com)**

Les parois cellulaires des racines sont le premier compartiment de la plante en contact avec les métaux, et vont influencer le transfert de Cu au sein de l'apoplasme et du symplasme. Ainsi le prélèvement des métaux intègre deux processus distincts : (i) l'adsorption des métaux dans l'apoplasme racinaire et à la surface des membranes plasmiques et (ii) l'absorption des métaux au travers des membranes plasmiques.

1.2.1 Adsorption du cuivre dans l'apoplasme racinaire

Le compartiment apoplasmique se subdivise en trois couches successives : la lamelle moyenne, la paroi primaire et la paroi secondaire qui est en contact avec la membrane plasmique (Sattelmacher 2001). Cette matrice est principalement constituée de cellulose, d'hémicellulose, de pectines et de protéines. L'apoplasme racinaire présente la particularité d'être chargé négativement, ce qui est principalement dû à la prédominance des fonctions carboxyliques dans la lamelle moyenne et la paroi primaire (Sattelmacher 2001). Sa composition joue un rôle important dans la complexation des métaux, les parois cellulaires se comportant comme des échangeurs d'ions spécifiques (Allan and Jarrell 1989). Cu peut alors être fixé sous forme cationique par les charges négatives des parois cellulaires. Cu peut aussi être lié sous forme non ionique par des réactions de coordination aux groupements contenant de l'azote, aux phosphatases et peroxydases également présents dans les parois cellulaires. L'apoplasme racinaire forme l'un des principaux compartiments accumulant les métaux (Krzeslowska 2011).

1.2.2 Absorption racinaire

Les mécanismes d'absorption de Cu restent à l'heure actuelle encore mal identifiés et ont été essentiellement caractérisés chez *Arabidopsis thaliana* L. (Burkhead et al. 2009; Puig et al. 2007b; Sancenon et al. 2004; Sancenon et al. 2003; Yruela 2009). L'absorption racinaire de Cu semble principalement dépendre de transporteurs à haute affinité spécifique de Cu(I) appartenant à la famille CTR (Copper TRansporter) et en particulier COPT1 (COPper Transporter protein), fortement exprimé dans les racines (Sancenon et al. 2004). L'expression de ce transporteur est contrôlée négativement par le cuivre (Sancenon et al. 2004). Ces protéines COPT transportent le cuivre sous une forme réduite Cu(I), mais celui-ci est majoritairement présent sous forme oxydée Cu(II) dans la solution de sol. La réduction du cuivre pourrait être assurée par des protéines réductases ferreuses (FRO 2 et FRO 3) présentes dans la membrane plasmique, qui sont impliquées dans la réduction du Fe^{3+} chez les dicotylédones (Mukherjee et al. 2006; Welch et al. 1993). Cependant la présence de ces réductases chez les monocotylédones n'est pas confirmée. En plus de l'import de Cu(I) par COPT1, Cu(II) pourrait être importé par des membres de la famille ZIP (Grotz and Guerinot 2006; Wintz et al. 2003). Cependant, le rôle de ces protéines dans le transport de cuivre *in planta* reste à vérifier.

1.3. Homéostasie au sein de la cellule

Il est primordial pour les cellules de contrôler finement l'homéostasie des métaux afin de pouvoir faire face à un excès de ces ions, et éviter des dommages tels que des stress oxydatifs. Une fois transportés dans les différents tissus, les métaux doivent être correctement distribués dans l'ensemble des compartiments subcellulaires et à des concentrations non toxiques pour la cellule. Ceci peut se faire grâce à la présence de transporteurs spécifiques et de molécules ou protéines qui vont prendre en charge ces métaux. Dans la cellule végétale, le cuivre est essentiel pour de nombreux processus comme la photosynthèse, la respiration, la perception de l'éthylène, le métabolisme des dérivés réactifs de l'oxygène.... Le cuivre doit donc être acheminé dans de nombreux compartiments subcellulaires comme le réticulum endoplasmique, la mitochondrie, le chloroplaste et l'apoplaste (Marschner 1995). Dans le cytosol, Cu(I) peut être chélaté par des phytochélatines et des métallothionines (Cobbett and Goldsbrough 2002) ou pris en charge par des métallochaperonnes. Les métallochaperonnes permettent d'acheminer le métal à une protéine cible spécifique via des interactions protéine-protéine. Ces métallochaperonnes permettraient aussi d'augmenter la capacité de la cellule à chélater Cu(I). Chez *Arabidopsis* (Figure I.3), deux protéines ont été identifiées : CCH (Copper CHaperone) et ATX1 qui interagiraient avec les ATPases HMA7 et HMA5 (Andrés-Colás et al. 2006). Différents facteurs entraînent une augmentation de leur expression comme une carence en cuivre, un

stress oxydatif, la sénescence ou encore des stress mécaniques (Burkhead et al. 2009). Récemment, deux nouvelles métallochaperonnes qui complexent Cu(II), CUTA et PCaP1, ont été identifiées et caractérisées (Burkead et al., 2003). CUTA est localisée dans le chloroplaste, PCaP1 serait associée à la membrane plasmique avec une forte affinité pour Cu(II). Elle ne possède pas de résidus cystéine ou histidine mais de nombreux résidus glutamate qui pourraient être impliqués dans la liaison du cuivre (Nagasaki-Takeuchi et al. 2008).

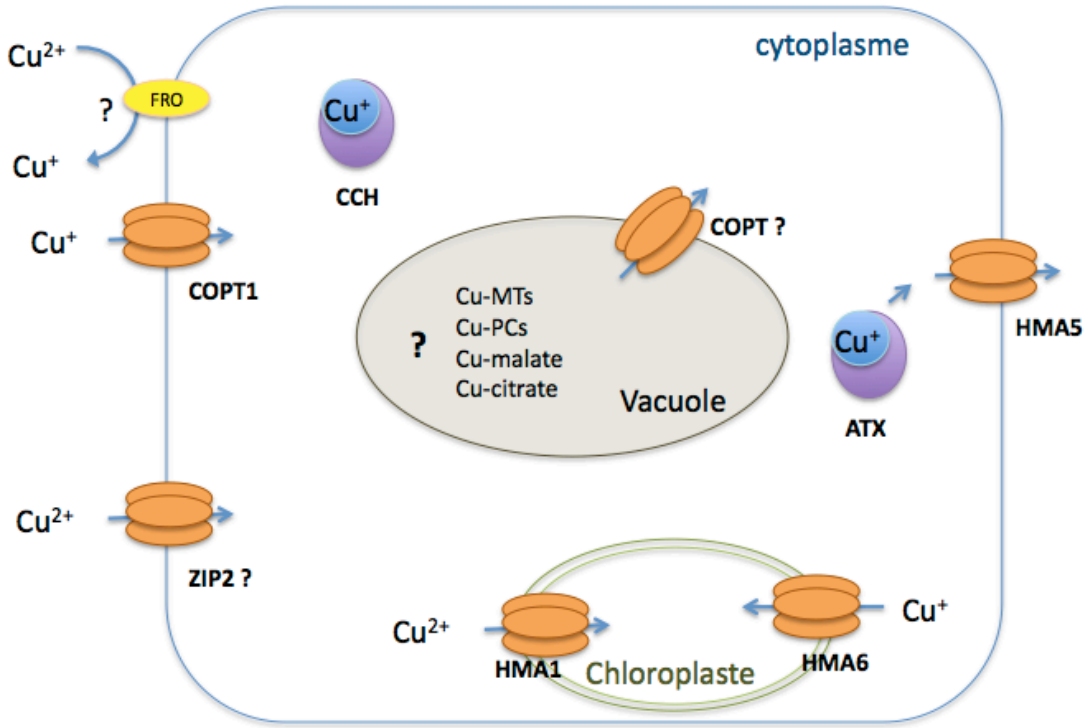


Figure I.3 **Schéma récapitulant les différents transports de Cu identifiés dans une cellule générique (essentiellement dans Arabidopsis). Les protéines membranaires transporteurs de Cu sont représentées en orange, les Cu chaperonnes en violet. Abréviations : CCH, copper chaperone; ATX : antioxydant1 ; COPT, copper transporter; FRO, ferric reductase oxidase; HMA, heavy metal P-type ATPase; MT, metallothioneins; ZIP, IRT-like protein. Schéma modifié d'après (Yruela 2009).**

1.4. Transport et complexation

Une fois incorporé dans les cellules racinaires, le cuivre est libéré dans les vaisseaux conducteurs de la sève brute, le xylème, pour être transporté jusqu'aux parties aériennes de la plante. L'efflux de Cu(I) de la racine vers le xylème, s'effectue en partie par le transporteur HMA5 (ATPase de type P) (Andrés-Colás et al. 2006). Cela a été en partie montré par la présence

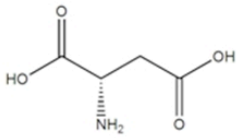
de HMA5 dans les racines et un blocage de la translocation de Cu, qui s'accumule alors dans les racines, dans un mutant déficient en HMA5.

Dans la sève, Cu peut modifier sa spéciation et former des complexes avec les substances organiques présentes (Pohlmeier 1999). Les ligands potentiels sont les suivants :

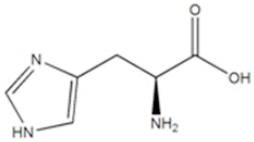
- carboxylates (citrate, oxalate, malate, succinate, tartrate, phtalate, salicylate, acétate),
- acides aminés et acides mercaptiques, ils possèdent au moins un groupement carboxylique et un groupement amine (glycine, acide glutamique, histidine, cystéine, asparagine, nicotianamine)(Figure I.4) (White et al. 1981a; White et al. 1981b),
- polymères (protéines, pectines, ADN, ARN, lignine et polysaccharides). Ils sont formés d'une chaîne de monomères qui possèdent des groupements fonctionnels carboxyliques ou aminés.

Le cuivre est distribué dans les différents organes, puis peut être remobilisé, c'est à dire changer d'organe en passant par les vaisseaux conducteurs de sève élaborée, le phloème.

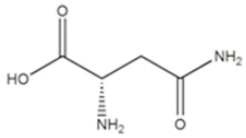
Exemples d'acides aminés



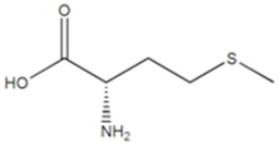
Asparate



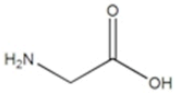
Histidine



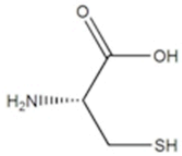
Asparagine



Méthionine

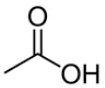


Glycine

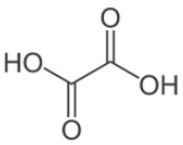


Cystéine

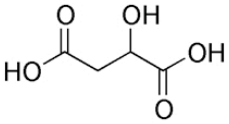
Exemples d'acides carboxyliques



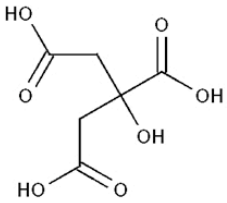
Acétate



Oxalate



Malate



Citrate

Structure chimique de la phytochélatine (a) et du glutathion (b)

(issue de (Pal and Rai 2010))

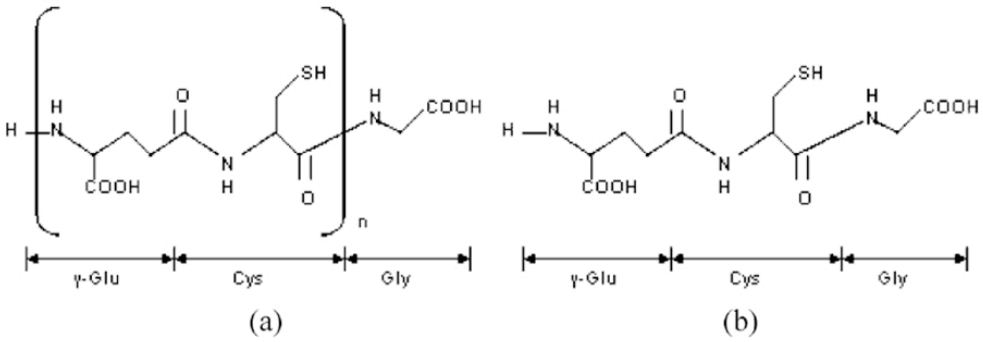


Figure I.4 **Structure chimique d'acides aminés, d'acides carboxyliques et de la phytochélatine et du glutathion**

1.5. Distribution

Dans la plupart des plantes étudiées, les concentrations racinaires de Cu sont supérieures aux concentrations des parties aériennes, que ce soit pour des plantes capables de tolérer de fortes quantités de Cu comme *Haumaniastrum katangense, Nicotiana plumbaginifolia, Elsholtzia haichowensis, Silene vulgaris* (Chipeng et al. 2009; Xia and Shen 2007), ou pour des plantes non tolérantes, comme le blé, le maïs, les roseaux, le niébé etc… (Ali et al. 2002; Bravin et al. 2010; Kopittke and Menzies 2006). Ainsi, le rapport des concentrations totales de Cu entre les racines et les parties aériennes peut considérablement varier et être compris entre 2.5 et 166 en fonction du niveau d'exposition des plantes (Brun et al. 2001; Santibanez et al. 2008). Cette forte accumulation racinaire s'explique en partie par la quantité importante de Cu liée aux parois cellulaires et/ou à un mécanisme de séquestration du cuivre. En séparant les parois cellulaires du cytoplasme de cellules de racines de *Athyrium yokoscense,* Nishizono et al. (1987) montrent que 70 à 90 % de Cu de la racine est localisé dans les parois cellulaires. Par une méthode similaire, Lou et al. (2004) mesurent 60 % du cuivre racinaire lié aux parois cellulaires dans *Elsholtzia haichowensis*. Ceci est également observé par observations in situ, comme par exemple dans la récente étude de Kopittke et al. (2011) ou l'on observe une forte accumulation du Cu sur les cellules de l'épiderme racinaire (Figure I.5).

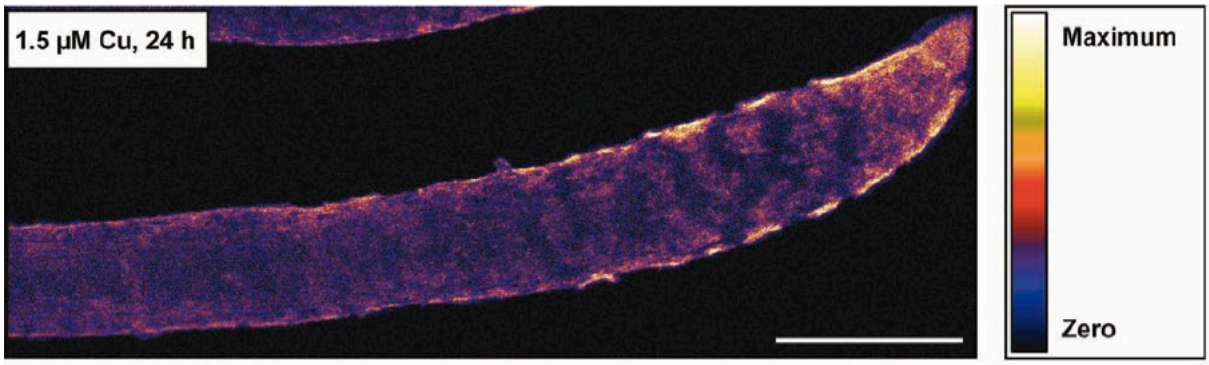


Figure I.5 **Distribution du cuivre sur une racine de niébé (cowpea) observée par µ-fluorescence X (Kopittke et al. 2011)**

1.6. Toxicité

L'origine des concentrations en Cu des sols est souvent multiple avec d'une part une origine naturelle ou fond pédogéochimique naturel et d'autre part une origine anthropique suite à des apports atmosphériques ou agricoles (épandages de fumier, de lisier, de boues de station d'épuration, de compost urbain, et l'utilisation de produits de traitement phytosanitaires

(Kabata-Pendias and Pendias 2001; Pilon-Smits 2005)). Au-delà de la concentration en Cu requise pour une croissance optimale, Cu devient toxique, excepté pour quelques plantes tolérantes à de fortes concentration de Cu (i.e. *Arabidopsis halleri* L. *Silene vulgaris* (Moench)., *Elshotzia splendens*) (Li et al. 2009; Qian et al. 2005; Shi et al. 2008; Shi et al. 2004; Song et al. 2004; Yang et al. 2002).

1.6.1 Rhizotoxicité

Un excès de Cu inhibe la croissance racinaire avant d'inhiber la croissance des tiges. Comme nous l'avons vu auparavant, Cu a une très forte affinité pour les parois cellulaires, et a donc tendance à s'accumuler dans ce compartiment (Iwasaki et al. 1990). Une inhibition de l'élongation racinaire et les dégâts causés à la membrane plasmique sont des symptômes immédiats d'une toxicité au Cu (De Vos et al. 1991). L'excès de Cu entraîne également certaines modifications morphologiques tel qu'un épaississement de la racine, une coloration brunâtre, une diminution de l'élongation racinaire et une augmentation de la ramification (Kopittke and Menzies 2006; Kopittke et al. 2009; Panou-Filotheou and Bosabalidis 2004). Ces symptômes sont principalement attribués à la rigidification des parois cellulaires par l'adsorption massive des métaux en remplacement du Ca dans l'apoplasme, en particulier au niveau de la zone d'élongation. Cette rigidité de l'apoplasme freine l'élongation racinaire et peut provoquer des ruptures au niveau des cellules du rhizoderme et du cortex externe qui seraient dues à des vitesses de croissance différentes entre les cellules des cortex internes et externes (Kopittke et al. 2008).

La concentration en Cu racinaire peut être une indication de la tolérance au Cu des plantes plus fiable que celle qui est donnée par les parties aériennes (Marschner 1995). En effet, la translocation de Cu aux parties aériennes est limitée par la forte accumulation de Cu dans les racines, donc les phénomènes de toxicité et les atteintes physiologiques (i.e. réduction de la croissance racinaire et de l'absorption des nutriments) se produisent avant que les parties aériennes n'atteignent des teneurs en Cu anormalement hautes (Michaud et al. 2008). Ainsi, Michaud et al. (2008) ont déterminé un seuil de 100-150 mg Cu kg^{-1} dans les racines, en incluant le compartiment apoplasmique, pour une rhizotoxicité modérée (EC10) et un seuil de 250-300 mg Cu kg^{-1} pour une rhizotoxicité importante (EC50).

1.6.2 Stress oxydatif et génotoxicité

Une concentration trop élevée de Cu induit la formation d'espèces réactives de l'oxygène (ROS) par la réaction d'Haber-Weiss et de Fenton. Cette formation d'ions superoxydes (oxygène

moléculaire ionisé par addition d'un électron supplémentaire) entraîne des dommages oxydatifs. Les plantes ont développé des systèmes de défense antioxydants qui, dans des conditions non ou peu stressantes, sont suffisants pour assurer l'homéostasie et ainsi empêcher des dégâts dus aux stress oxydatifs (Foyer et al. 1994). Les enzymes antioxydantes présentes en majorité sont des enzymes détoxifiantes : superoxides dismutases (SOD), Cu/Zn SOD, catalase (CAT), et des enzymes peroxydases qui décomposent les composés péroxydes (H_20_2), comme les glutathions peroxydases. De nombreux antioxydants non-enzymatiques sont également impliqués : le glutathion, l'acide ascorbique, les caroténoïdes, l'acide urique, plusieurs acides aminés et des thiols.

Quand le système ne suffit plus à empêcher le stress oxydatif, les ROS par leur nature instable, sont particulièrement réactives et sont capables de provoquer des dégâts cellulaires importants (Briat and Lebrun 1999; Dietz and Baier 1999):

- la peroxydation des membranes lipidiques ; les acides gras polyinsaturés des membranes cellulaires ou des lipoprotéines sont la cible principale des ROS (Luna et al. 1994),
- cassures et mutations de l'ADN (Lloyd et al. 1997),
- inactivation des protéines et des enzymes,
- oxydation des pigments, des sucres…,
- dégradation de la chlorophylle et inhibition de la photosynthèse (Luna et al. 1994; Patsikka et al. 2002).

Le cuivre peut également être génotoxique, c'est à dire capable de générer des mutations génétiques (génique, chromosomiques ou génomiques). Les effets génotoxiques sont vraisemblablement causés par les radicaux libres et agents oxydants libérés par l'action du métal (Souguir et al. 2008; Yildiz et al. 2009).

1.6.3 Phytotoxicité au niveau des parties aériennes

La phytotoxicité au niveau des parties aériennes s'observe par une réduction de biomasse, des symptômes chlorotiques, des nécroses et une inhibition de la croissance. Une diminution de la quantité de chlorophylle, des altérations de la structure des chloroplastes et de la composition de la membrane des thylakoïdes ont été observées dans le riz, le blé, le haricot et l'origan dans des conditions toxiques (Panou-Filotheou and Bosabalidis 2004; Patsikka et al. 2002; Yruela 2009). Il a été proposé que Cu interfère avec la biosynthèse de l'appareil photosynthétique, modifiant la composition des pigments et de la membrane interne des chloroplastes (Maksymiec

et al. 1994). Patsikka et al. (2002) attribuent cette réduction de chlorophylle à une déficience en Fe induite par la toxicité de Cu. Comme on l'a signalé dans le paragraphe précédent, cela peut également être dû au stress oxydatif qui entraîne la dégradation des membranes des thylakoides (Luna et al. 1994).

La phytotoxicité de Cu semble également se traduire par l'apparition de déficiences en nutriments. Des diminutions significatives des concentrations en Ca, Fe, K, Mg, Mn et Zn dans les parties aériennes de Rhodes Grass, du niébé (cowpea), et du maïs ont été observées (Ait Ali et al. 2002; Kopittke and Menzies 2006; Sheldon and Menzies 2005). Des décolorations internervaires, synonymes de chlorose ferrique, ont également été décrites. L'origine exacte de ces phénomènes de déficience n'est pas connue précisément. Cependant, l'apparition simultanée de déficiences multiples laisse penser qu'il s'agit d'une conséquence de l'altération de la structure du système racinaire, comme la dégradation de la membrane plasmique (Kopittke et al. 2009; Panou-Filotheou and Bosabalidis 2004). Dans le cas particulier des monocotylédones, l'induction d'une déficience en Fe lors d'une forte exposition à Cu pourrait être due à un mécanisme plus spécifique, impliquant la perturbation par Cu^{2+} du rôle complexant des phytosidérophores vis-à-vis de Fe^{3+}, ou à un antagonisme Fe-Cu au niveau des transporteurs des complexes formés par les phytosidérophores avec ces deux métaux (Michaud et al. 2008).

1.7. Mécanisme de tolérance à la toxicité de Cu

Les mécanismes cellulaires impliqués dans la tolérance au cuivre peuvent avoir les effets suivants (Clemens 2006; Hall 2002; Yruela 2009) :

- réduire l'absorption métallique via l'action des mycorhizes ou des exsudats extra cellulaires,
- immobiliser l'excès de Cu dans les racines et ainsi exclure le métal des parties aériennes,
- stimuler la pompe à efflux du métal dans la membrane plasmique,
- chélater Cu par des phytochélatines, des métallothionines, des acides organiques ou des protéines de choc thermique (HSP),
- séquestrer Cu dans les vacuoles.

Des acides organiques (citrate, malate, oxalate), carbohydrates, protéines ou peptides sont excrétés par les plantes et peuvent faciliter l'absorption des métaux, mais ces molécules peuvent également inhiber leur acquisition en formant des complexes extérieurs à la racine. L'importance de ces mécanismes semble varier selon la concentration de métaux, les espèces et variétés impliquées et le temps d'exposition. Des dépôts foncés extracellulaires riches en Cu, couplés à une forte concentration de citrate et de malate ont été observés sur des cellules isolées

de soja, en présence de 10 µM de Cu (Bernal et al. 2006). Des concentrations similaires de citrate et de malate ont été observées dans des cellules tolérantes au Cu de *Nicotiana plumbaginifolia* L. (Kishinami and Widholm 1987). Le rôle des acides organiques dans la rhizosphère est important pour tolérer une contamination aluminique (Jones 1998), même si les mécanismes qui activent et régulent la synthèse des acides organiques ne sont toujours pas bien identifiés (Walker et al. 2003).

Une fois internalisés, les métaux en excès vont être stockés dans un endroit métaboliquement peu actif. Une séquestration des métaux peut avoir lieu dans la vacuole, l'apoplasme, ou dans des cellules spécialisées comme les cellules épidermales ou des trichômes.

Malgré la présence de métallothioneines (MTs) et la forte expression de certains gènes des MTs, leurs fonctions dans les plantes restent peu connues. L'expression de plusieurs gènes des MTs est induite par la présence de Cu (Guo et al. 2003), le niveau d'expression du gène du type 2 de MTs est associé à la tolérance au Cu chez les *A thaliana* (Murphy and Taiz 1995) et la tolérance des plantes métallophytes *Silene vulgaris* et *Silene paradoxa* est associée à une augmentation de l'expression des gènes MTs (van Hoof et al. 2001). Cependant, l'implication des MTs dans la détoxification de Cu dans les plantes n'a pas été démontrée de manière concluante (Hassinen et al. 2011). La divergence des séquences des protéines MTs et le profil d'expression complexe des gènes MTs laisse suggérer que les fonctions de MTs ne sont pas limitées à la détoxification de Cu. Récemment, des preuves ont été apportées que les MTs contribuent à l'homéostasie des métaux dans les plantes (Guo et al. 2008; Roosens et al. 2004).

Le rôle des phytochélatines (PCs) dans la détoxification de Cu n'a pas été mis en évidence. Cu est un activateur de la biosynthèse de PCs, mais les plantes mutantes déficientes en PCs montrent peu de sensibilité au cuivre (Lee and Kang 2005; Schat et al. 2002). Comme les PCs peuvent former des complexes avec Cu, il est possible que les complexes PC-Cu ne soient pas séquestrés dans la vacuole (Cobbett and Goldsbrough 2002). Récemment Singh et al. (2010) montrent aussi que la production de PCs n'est pas liée à la tolérance de Cu dans le pois chiche (*Cicer arietinum*).

Le métabolisme de l'azote a un rôle central dans la réponse des plantes au stress métallique (Lea and Azevedo 2007). L'accumulation de certains acides aminés, comme la proline ou l'histidine a été mesurée dans des plantes ayant été soumises à de fortes teneurs de Cu laissant suggérer un rôle en tant que chélateur ou antioxydant (Sharma and Dietz 2006).

Les ATPases de types P1B qui transportent Cu sont importantes, pas uniquement pour importer les quantités de Cu nécessaires au fonctionnement de la cellule, mais aussi pour limiter leur accumulation. Le cuivre est chélaté à des chaperonnes qui le transportent jusqu'aux organelles

ou directement à des protéines cytosoliques dépendantes de Cu. Il semble que la chélation et le transport de Cu ne soit pas seulement requis pour l'absorption de Cu mais aussi pour des processus de détoxification (Puig et al. 2007). Par exemple, l'inactivation du gène ActP, qui encode une ATPase de type P entraîne une hypersensibilité du *Rhizobium legominosarum* et du *Sinorhizobium meliloti* (Reeve et al. 2002). Il a été montré que *chez A. thaliana* le transporteur ATPase HMA5 améliore la détoxification de Cu (Andrés-Colás et al. 2006).

1.8. Utilisation de la spectroscopie d'absorption des rayons X pour étudier la spéciation de Cu dans les plantes

La spectroscopie d'absorption des rayons X est utilisée pour caractériser la spéciation des éléments traces métalliques dans les plantes (Kramer et al. 2000; Salt et al. 2002; Sarret et al. 2009). L'utilisation de la XAS présente de nombreux avantages comme l'absence de prétraitement de l'échantillon, qui est susceptible de modifier la spéciation de l'élément étudié (Gardea-Torresdey et al. 2005). Les méthodes basées sur des extractions séquentielles ou sur une homogénéisation des tissus frais (par exemple la chromatographie) entraînent une dégradation des membranes intracellulaires. Les métaux faiblement liés initialement localisés dans les vacuoles, peuvent se retrouver en contact avec divers ligands forts du cytoplasme, entraînant alors des changements artificiels de la spéciation. Parmi les autres avantages de cette technique spectroscopique, nous pouvons citer la sélectivité chimique ainsi que les seuils de détection bas qui permettent de travailler avec des concentrations physiologiques. La Figure I.6 résume les principales informations que l'on peut obtenir grâce à l'analyse d'un spectre d'absorption des rayons X.

La spéciation du cuivre (état d'oxydation, nombre et nature des voisins) dans les tissus des plantes est peu étudiée malgré une bonne connaissance de la structure et des fonctions de nombreuses enzymes qui dépendent du cuivre et des Cu chaperonnes (voir la review de Pilon par exemple (2006)).

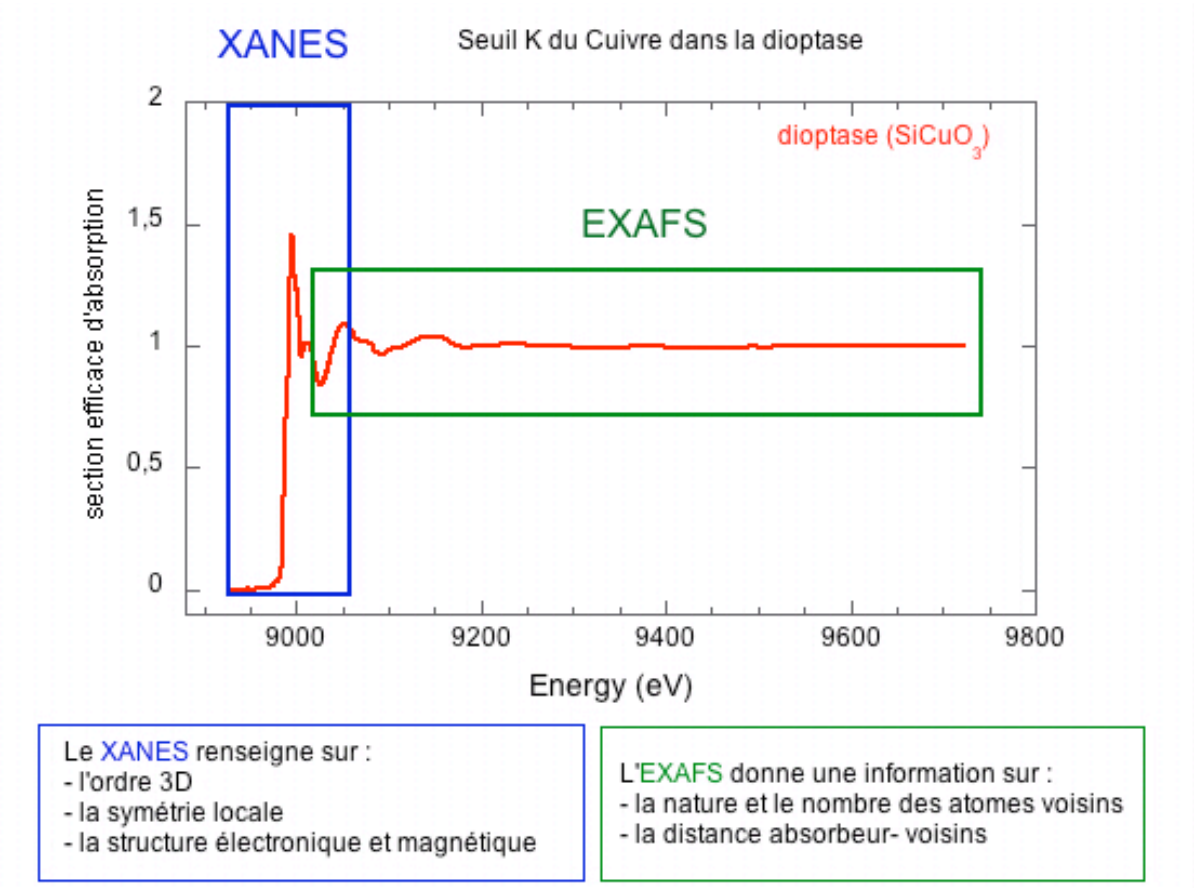


Figure I.6 Schéma présentant les caractéristiques du XANES et de l'EXAFS

La première étude décrivant la spéciation du cuivre dans une plante a été réalisée par Polette et al. (2000) dans *Larrea tridentata* (créosotier). Ces plantes ont accumulé des concentrations élevées de Cu : 953 $mg.kg^{-1}$ dans les racines, 493 $mg.kg^{-1}$ dans les tiges et 370 $mg.kg^{-1}$ dans les feuilles. Après l'échantillonnage des plantes, les échantillons ont été immédiatement congelés dans l'azote liquide. L'analyse XAS a ensuite été effectuée avec un cryostat à hélium. La préparation des échantillons et leur analyse à froid répond à un double enjeu : i) éviter de modifier la spéciation *in situ* qui peut être provoquée par une altération des structures, lors du séchage par exemple, ii) limiter au maximum les dommages dus au faisceau de photons sur l'échantillon. Dans un échantillon organique, le cuivre peut être réduit en Cu(I) ou Cu(0) par les électrons, radicaux libres, H_2 ou autres espèces réactives de l'oxygène qui ont été formés par la radiolyse de l'eau suite à l'effet ionisant des rayons X (Manceau and Matynia 2010; Mesu et al. 2006). Une manière de retarder l'apparition de ces dégâts est d'analyser l'échantillon à froid (Manceau et al. 2002).

L'observation du spectre XANES du sol, des racines, de la tige et des feuilles (Figure I.7) permet de décrire l'état d'oxydation de Cu. La position de la raie blanche (environ 8998 eV) indique

dans les trois parties de la plante et dans le sol la présence de Cu(II). L'épaulement situé autour de 8984 eV est caractéristique des composés Cu(I). Les auteurs observent un épaulement de plus en plus marqué de la racine aux feuilles, et suggèrent donc que la réduction du Cu(II) en Cu(I) a lieu lors du transport dans la plante.

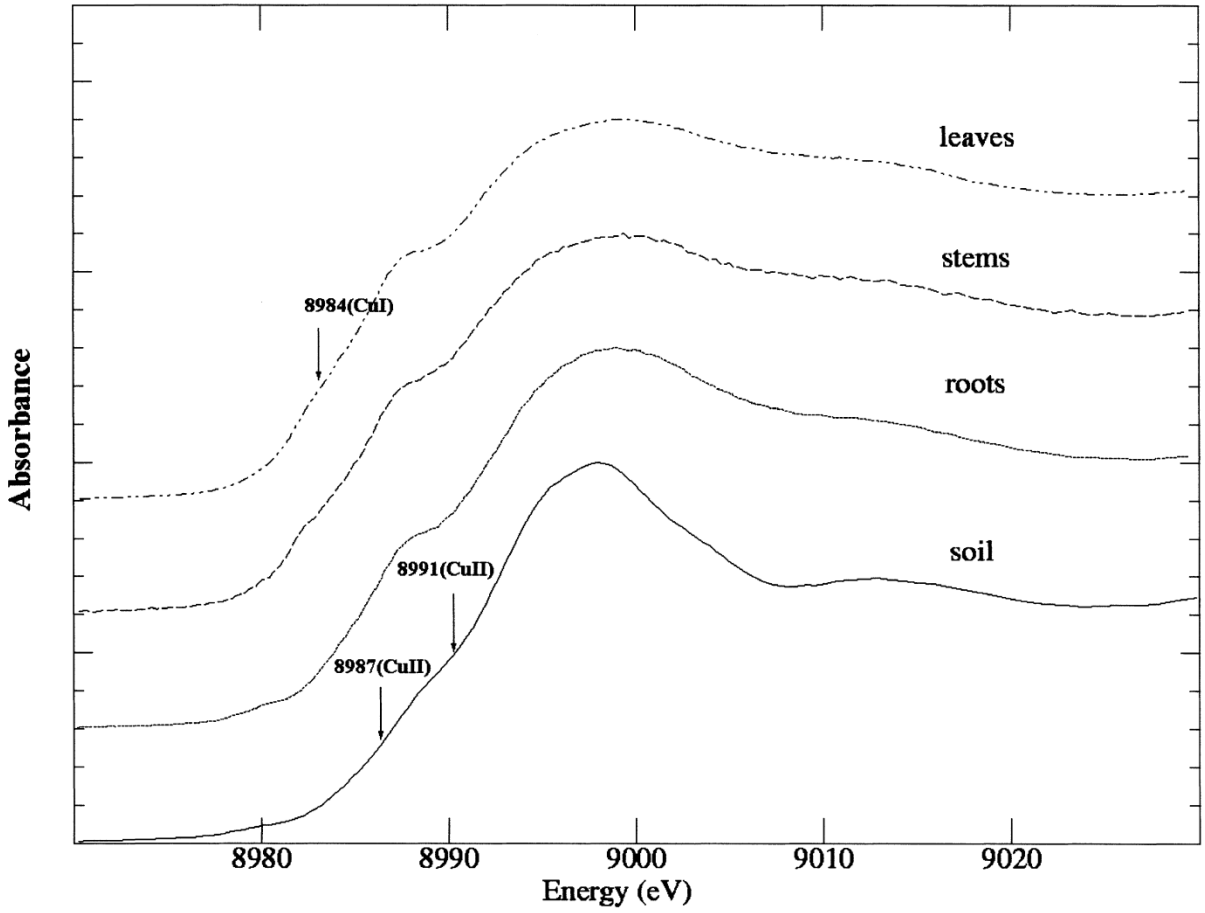


Figure I.7 Spectre XANES au seuil K du Cu des feuilles, tiges, racines de créosotier et du sol d'origine des plantes (Polette et al. 2000)

L'affinement des spectres EXAFS montre la présence de liaisons de type Cu-O (1,83 Å et 1,90 Å) et Cu-S (2,24 et 2,27 Å) dans les racines, tiges et feuilles. Ils proposent également l'existence d'interactions Cu-Cu de 2,78 Å dans les feuilles et les tiges et une interaction Cu-Cu de 3,70 Å dans toute la plante.

Les auteurs font alors les hypothèses suivantes :

- une phase de stockage : Cu-phytochélatines,
- une phase précipitée, soit formée dans la plante (ex chalcopyrite) ou d'origine extérieure et internalisée par le biais des stomates.

Une autre étude de Gardea-Torresdey et al. (2001) a également porté sur la spéciation de Cu dans le créosotier, mais en utilisant des plantes développées en culture hydroponique, exposées à une concentration de 635 $mg.L^{-1}$ de $CuSO_4$. Les plantes ont été mises au contact d'une solution de $CuSO_4$ pendant 48h. La préparation des plantes précédant l'analyse XAS n'est pas détaillée, il semble que les différentes parties de la plante ont été séchées à 59°C. Contrairement à la précédente étude, Cu est présent uniquement sous forme oxydée dans les tiges, racines et feuilles et est complexé à un ligand de type acide organique. Un résultat similaire est observé chez la plante *Sesbania drummondii* (Sahi et al. 2007) qui a été cultivée en hydroponie et exposée à des concentrations de 25 $mg.L^{-1}$ et 100 $mg.L^{-1}$ de $CuSO_4$ pendant 10 jours. Préalablement à l'analyse XAS, les échantillons ont été lyophilisés. Les auteurs ne précisent pas quelle partie de la plante est analysée, nous supposons qu'il s'agit de la plante entière. Le cuivre présente un seul degré d'oxydation Cu(II) dans la plante. Des analyses par combinaisons linéaires montrent que Cu est complexé à des ligands semblables à des sucres ou des acides organiques (référence utilisée : Cu(II)-gluconate) et une forme précipitée (Cu(II)oxide et Cu(II)nitrate). La modélisation des spectres EXAFS indique la présence de 4 voisins O à 1.95 Å , 2 voisins O à 2,32 Å, et 4 voisins C à 2,80 Å.
Cependant dans ces deux études, la spéciation de Cu peut avoir été influencée par le séchage de l'échantillon. Le séchage peut entraîner une oxydation de Cu présent dans les tissus et éventuellement la précipitation de la phase décrite par Sahi et al (2007) dans leurs échantillons.

Shi et al (2008) ont étudié la spéciation de Cu présent d'une plante tolérante Elshotzia splendens qui a été cultivée en hydroponie et mise au contact d'une solution de 300 µM de Cu pendant 10 ou 60 jours. Les concentrations en Cu des tissus varient entre 165 et 248 $mg.kg^{-1}$ dans les feuilles, 202 à 263 $mg.kg^{-1}$ dans les tiges et 11 755 et 13 796 $mg.kg^{-1}$ dans les racines. Les échantillons frais ont été congelés à l'azote liquide. La spéciation de Cu est similaire dans les feuilles, tiges et racines et est caractérisée par une forte proportion de Cu(II), même si la présence de Cu(I) est détectée. L'analyse par combinaison linéaire des spectres XANES indique que les espèces de type : Cu-histidine (33-52 %) et Cu-parois cellulaires sont majoritaires, alors que les espèces de type Cu-oxalate (9-18 %) et Cu-glutathione (7-16 %) sont minoritaires. D'après la modélisation des spectres EXAFS, l'interaction dominante est Cu-N/O à 1,94-1,97 Å dans les racines, les tiges et les feuilles.

Ainsi, les mécanismes de tolérance de Cu dans *E. Splendens* impliqueraient :

- La complexation de Cu sur les parois cellulaires, notamment sur les groupes oxygénés -OH ou –COOH présents sur la cellulose, la lignine ou la pectine,
- La complexation à des acides aminés (de type histidine) ou organiques, éventuellement séquestrés dans la vacuole,

- Une absence de complexation à des composés de type phytochélatines.

Deux études récentes ont étudié la complexation et la toxicité du cuivre dans deux plantes : une plante accumulatrice de Cu *Crassula helmsii* et une plante non tolérante *Thlaspi caerulescens* (Kupper et al. 2009; Mijovilovich et al. 2009).

Des graines de *T. caerulescens* ont été placées en culture hydroponique et soumises à 10 µM de Cu pendant 8 semaines. Certaines plantes dites « résistantes » se sont mieux adaptées à l'apport de Cu et ont été cultivées pendant 4 mois. Les concentrations en Cu des feuilles des plantes dites « sensibles » sont de 31,4 $mg.kg^{-1}$ MS et celles des plantes « résistantes » de 42,7 $mg.kg^{-1}$ MS. L'affinement des spectres EXAFS (Figure I.8) indique la présence de ligands O et S dans la première sphère de coordination et des interactions Cu-Cu à plus grande distance. Une partie de ligand O ayant une contribution à 2,8 Å proviendrait de la nicotianamide. La présence de Cu a été proposée car l'interaction Cu-Cu expliquerait le pic observé sur la transformée de Fourier à 4,4 Å et à 5,2 Å. L'analyse par combinaison linéaire indique la présence de ligands de type : soufré (Cu(I/II)-glutathione), acide organique (CuSO4, Cu(II)-malate, Cu(II)-citrate, Cu(II)-Pro), nicotianamine, histidine, dont les proportions varient selon la résistance à Cu et l'âge des feuilles (juvéniles ou matures).

Ainsi, les auteurs suggèrent :

- la présence de Cu lié à des métallothioneines, en quantité plus importante dans les individus résistants,
- de la biominéralisation de Cu. Cela pourrait être expliqué en partie par la présence de moolooite, une forme hydratée cristalline de Cu oxalate, détectée auparavant dans du lichen, et une autre forme, comme un oxyde de Cu, ou une hydrocalcite ($CaCO_3.H_2O$),
- La présence de Cu lié à de la nicotianamide.

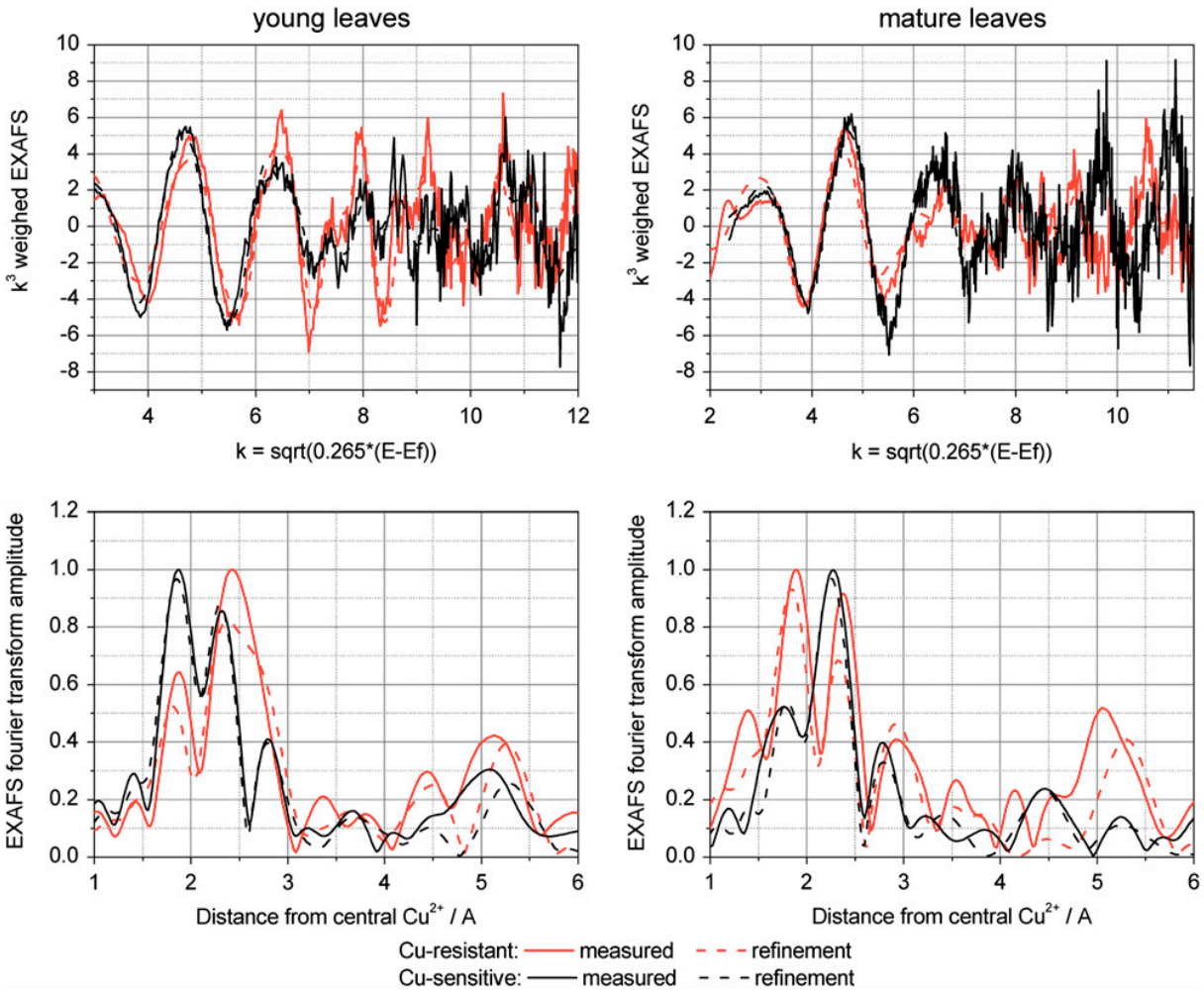


Figure I.8 **Spectres EXAFS normalisés et transformées de Fourier des échantillons de feuilles de *Thalspi caerulescens* de l'étude de Mijovilovich et al (2009). Les données sont représentées en traits pleins et les fits en pointillés.**

La seconde étude réalisée par la même équipe (Kupper et al. 2009) présente la spéciation de Cu dans une plante amphibienne *Crassula helmsii* qui semble tolérer de fortes quantités de Cu. Les quantités de Cu accumulées sont de 9200 et 9100 mg.kg^{-1} MS dans les parties émergées et immergées respectivement. L'analyse EXAFS des spectres de ces deux parties des plantes indique uniquement la présence de ligands oxygénés. Ils proposent donc la présence de ligands de type malate ou citrate.

D'après les résultats de ces deux études, les auteurs proposent l'existence de deux stratégies pour faire face à une toxicité de Cu :

- Séquestration de Cu dans les vacuoles ou les parois cellulaires, associé à des ligands acides organiques ou acides aminés dans les plantes accumulatrices.
- Détoxification par complexation de Cu à des PCs ou des MTs, minéralisation de Cu, dans les plantes non accumulatrices.

Enfin, Koppitke et al. (2011) ont étudié la localisation et la spéciation de Cu dans des racines de niébé (cowpea) ayant été mises au contact de 1,5 µM de Cu pendant une courte période (3h et 24h). Les concentrations racinaires sont égales à 96 $mg.kg^{-1}$ MS après 3h et 190 $mg.kg^{-1}$ MS après 24h. L'analyse par combinaisons linéaires des spectres XANES et EXAFS des plantes au contact de Cu pendant 3h, indique un mélange de différents ligands de Cu composé de 45 à 60 % de Cu-cysteine, de 40 à 55 % de Cu-citrate (EXAFS) ou de Cu-histidine (XANES) pour les racines. Après 24h, le Cu racinaire est majoritairement associé à 60 % d'acide polygalacturonique, et a de la cystéine ou du citrate (Figure I.9). Les auteurs concluent que Cu est principalement accumulé dans la paroi cellulaire, l'acide polygalacturonique étant le principal composant de la pectine, mais aussi lié à des composés thiol tels que PCs ou MTs.

Les auteurs ne s'interrogent pas sur la présence de Cu(I) dans l'échantillon. Pourtant, le complexe Cu-cystéine synthétisé peut contenir du Cu(I). Il apparaît cependant difficile de comparer les spectres XANES car le maximum d'absorption des spectres XANES de leurs échantillons est situé à une énergie autour de 8982 eV (Figure I.9), alors que la transition 1s-4p des composés Cu(II) est normalement située autour de 8995eV. On voit bien cependant que l'épaulement présent dans la cystéine est présent à plus faible énergie que l'épaulement de l'acide polygalacturonique. Ces transitions pourraient alors être 1s-$4p_{xy}$ du Cu(I) de la cystéine et 1s-$4p_z$ du Cu(II) de l'acide polygalacturonique. Il aurait été intéressant d'avoir les dérivées de ces spectres afin de mieux visualiser les transitions. Le Cu présent dans la racine au contact de Cu pendant 3h ayant une signature proche de la cystéine, pourrait donc contenir Cu(I).

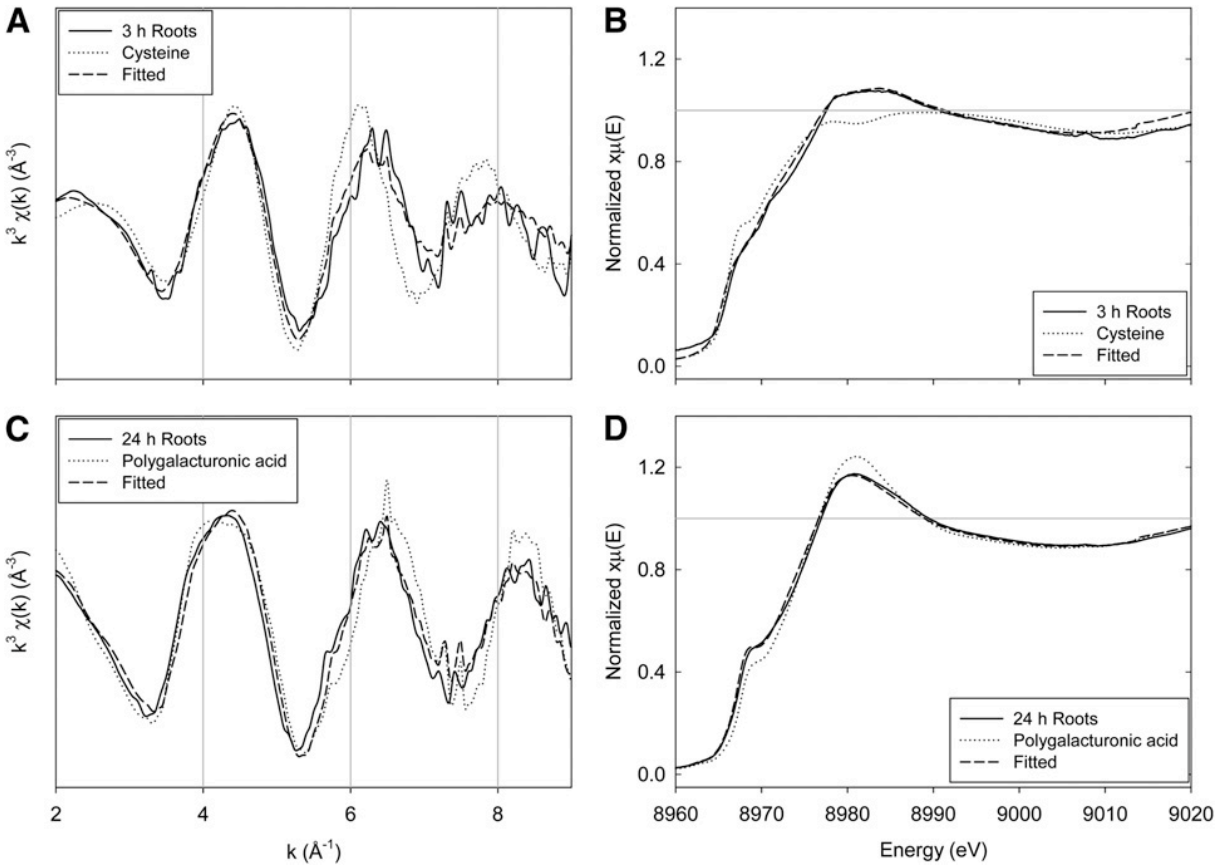


Figure I.9 **Spectres EXAFS (A et C) et XANES (B et D) au seuil K du Cu des racines de niébé (cowpea) soumises à 1,5 µM Cu pendant 3h (en haut) ou 24h (en bas). Les spectres des composés de référence les plus importants (i.e. acide polygalacturonique ou cystéine) et les résultats des combinaisons linéaires sont également représentés.**

Au vu de la description des quelques publications décrivant la spéciation de Cu dans les plantes, il apparaît difficile de comparer la spéciation entre les espèces. En effet, les résultats présentés varient en fonction du mode de préparation des plantes (congélation ou séchage), de la concentration de Cu du milieu, de la durée d'exposition, et peuvent aussi être influencés par les différentes méthodes d'analyse des spectres EXAFS (combinaison linéaire ou modélisation des différentes sphères de coordination).

2. LE SILICIUM

Le silicium (Si) représente le second élément le plus abondant dans le sol, après l'oxygène. Ainsi, la plupart des plantes se développant sur du sol contiennent de la silice dans leurs tissus (Epstein 1994). La question du rôle de Si dans le développement des plantes a été soulevée depuis plus d'un siècle (Epstein 1994; Jones and Handreck 1965; Sachs 1862). Les teneurs en Si dans les plantes sont comparables aux teneurs des macronutriments, mais le silicium n'est pas considéré comme un élément « essentiel » pour la croissance des plantes (Epstein 1994; Epstein 1999). La principale raison est l'absence de preuve de l'implication de Si dans le métabolisme de la plante, qui est l'un des trois critères requis pour l'essentialité établis par Arnon et Stout (1939). Cependant, de nombreuses études mettent en évidence le rôle bénéfique de cet élément pour les plantes : amélioration de la croissance, meilleure résistance aux stress biotiques et abiotiques (Ma and Takahashi 2002).

2.1. Teneur en Si dans les plantes et dans le bambou

Les teneurs d'acide silicique mesurées dans les plantes varient entre 1 et 100 $mg.g^{-1}$ de matière sèche (MS) en fonction de l'espèce (Epstein 1999; Hodson et al. 2005). Hodson et al. (2005) montrent que la concentration en Si des plantes dépend majoritairement de la position phylogénétique de l'espèce plutôt que de son environnement (i.e concentration en Si dans le sol et la solution de sol, pH, climat…). Les espèces les plus riches font partie de la famille des Poacées *(Poaceae)* dans la classe des monocotylédones. La capacité d'accumuler Si dans les parties aériennes dépend de l'absorption racinaire, qui n'a pas la même efficacité selon les espèces. Les plantes sont considérées comme « accumulatrices » quand la teneur en Si est supérieure à 10 $mg.g^{-1}$ (Ma and Takahashi 2002). Avec une concentration en Si pouvant atteindre jusqu'à 410 $mg.g^{-1}$ (Table I.1), les bambous font partie des plantes qui accumulent le plus de silicium (Motomura et al. 2002). Comme le montre la Table I.1, le nombre de publications présentant des teneurs dans les bambous reste faible et quelques questions importantes restent sujettes à controverse. Par exemple, Ding et al., (2008b) mesurent dans les racines une concentration de 7,9 $mg.g^{-1}$, concentration qui se situe entre celle des tiges (3,9 $mg.g^{-1}$) et des branches (17,8 $mg.g^{-1}$). Alors que Li et al.,(2006) et Lux et al., (2003) reportent des teneurs dans les racines supérieures aux feuilles : 73,2 $mg.g^{-1}$ et 24 $mg.g^{-1}$ respectivement.

Table I.1 Synthèse des concentrations en Si publiées dans le bambou

	Mean ± standard deviation		min	max	Number of samples	References
Leaves	94	± 75	20	410	22	1 to 6
Stems	8	± 8	3	44	22	1-2-6
Branches	17	± 4	8	20	8	1-6
Rhizomes	7	± 4	3	17	12	4-6
Roots	18	± 22	6	73	9	1-6

Les concentrations en Si sont exprimées en SiO_2 mg g^{-1} de matière sèche.
1: Ding et al. 2008; 2: Li et al. 2006; 3: Lux et al. 2003; 4: Meunier et al. 1999; 5: Motomura et al. 2002; 6: Ueda and Ueda. 1961 (from Ma and Takahashi, 2002).

2.2. Absorption racinaire

Dans le sol, il existe trois principaux réservoirs de silicium : (a) la silice et les minéraux siliceux (b), les phytolithes et (c) le silicium dissous dans la solution du sol (Sauer et al. 2006). La solution du sol est dominée par la présence d'acide silicique monomère, $Si(OH)_4$. La concentration d'acide silicique mesurée dans les solutions du sol varie de 0.1 à 0.6 $mmol.L^{-1}$ (Epstein 1994). Il a été suggéré dans de nombreuses études que les plantes absorbent Si sous cette forme. Dans le bambou, Ding et al. (2008b) confirment cela par une étude isotopique de Si dans le bambou, le sol et la solution du sol.

Les mécanismes d'absorption de Si au niveau de la racine (passage de la solution externe aux cellules corticales) font l'objet de controverses. Elle s'effectuerait par trois mécanismes : de la diffusion passive, un transport actif, mais aussi un processus d'exclusion (Ma et al. 2004; Mitani and Ma 2005). Les espèces dites « non accumulatrices » ont dans leurs tissus des teneurs en Si inférieures aux teneurs de la solution du sol. Elles ont développé un processus d'exclusion empêchant l'absorption de Si par diffusion passive (Raven 2001). Lors d'un processus d'absorption passif, la plante absorbe le silicium contenu dans la solution du sol selon le gradient de concentration. Le flux passif est principalement contrôlé par l'évapotranspiration. Cette hypothèse est basée sur le fait que les sites de précipitation de silice coïncident avec les principaux sites d'évapotranspiration (Ding et al. 2008a; Ding et al. 2008b). Le silicium tend à s'accumuler là où l'eau s'évapore le plus : feuilles les plus hautes, les plus exposées à l'ensoleillement (Motomura et al. 2002; Raven 2001; 2003). Dans le cas d'un processus actif, le silicium est absorbé grâce à des protéines membranaires spécifiques qui permettent à Si de s'accumuler dans la plante en dépit du gradient de concentration. Ce type de

transporteur est plus rapide que le transport de l'eau et indépendant de la transpiration. Rains et al., (2006) ont mis en évidence ce phénomène actif dans le blé (*Triticum aestivum*) en montrant que l'absorption de Si suit une cinétique de Michaelis-Menten. A l'aide de mutants, de nombreuses études réalisées par l'équipe de Ma et ses collègues (voir review (Ma et al. 2011)) ont identifié deux gènes responsables du transport et de l'absorption de Si dans plusieurs plantes (Figure I.11):

- Lsi1 : transporteur d'influx qui fait pénétrer le Si dans la cellule
- Lsi2 : transporteur d'efflux qui fait sortir le Si de la cellule

Le transporteur Lsi1 initialement découvert dans le riz a des homologues présents dans le blé, le maïs et la citrouille (curcurbitacée) (Chiba et al. 2009; Mitani et al. 2011; Mitani et al. 2009b). Le transporteur Lsi2 a été identifié dans le riz, le blé et le maïs (Mitani et al. 2009a). Cependant, des différences de localisation de ces transporteurs dans les cellules racinaires sont observées entre les espèces et pourraient être à l'origine des capacités d'accumulations différentes.

2.3. Transport et stockage

Après l'absorption racinaire, Si est transporté aux parties aériennes via le xylème. Le transporteur qui permet à Si d'entrer dans le xylème n'a pas été identifié. Une partie du silicium transporté dans le flux de sève va polymériser et former un gel de silice (SiO_2, nH_2O). En effet, lorsque la concentration de Si est supérieure à 2 mM, le processus de polymérisation convertit les monomères d'acide silicique en oligomères, puis en particules de silice formant des agrégats (Perry and Keeling-Tucker 2003) (Figure I.10).

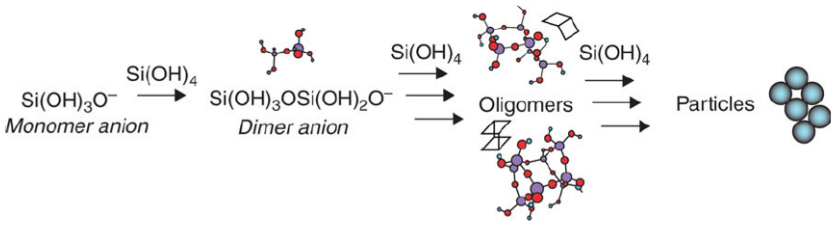


Figure I.10 **Processus de formation de particules de silice par différentes réactions de polymérisation (dimères, oligomères et agrégats) de monomères de silicium (Currie and Perry 2007).**

Quand les particules de silice se sont développées pour atteindre une taille comprise entre 1 et 3 nm (tailles observées dans la nature), les particules portent alors une charge de surface négative et interagissent avec l'environnement local, comme les parois cellulaires (Currie and Perry

2007). Malgré ces observations et la forte concentration de Si dans la sève du xylème, qui peut atteindre 18 mM, Si est majoritairement détecté sous une forme monomérique : H_4SiO_4 (Casey et al. 2004; Ding et al. 2005; Mitani et al. 2005). Mais le mécanisme empêchant la polymérisation n'est pas connu.

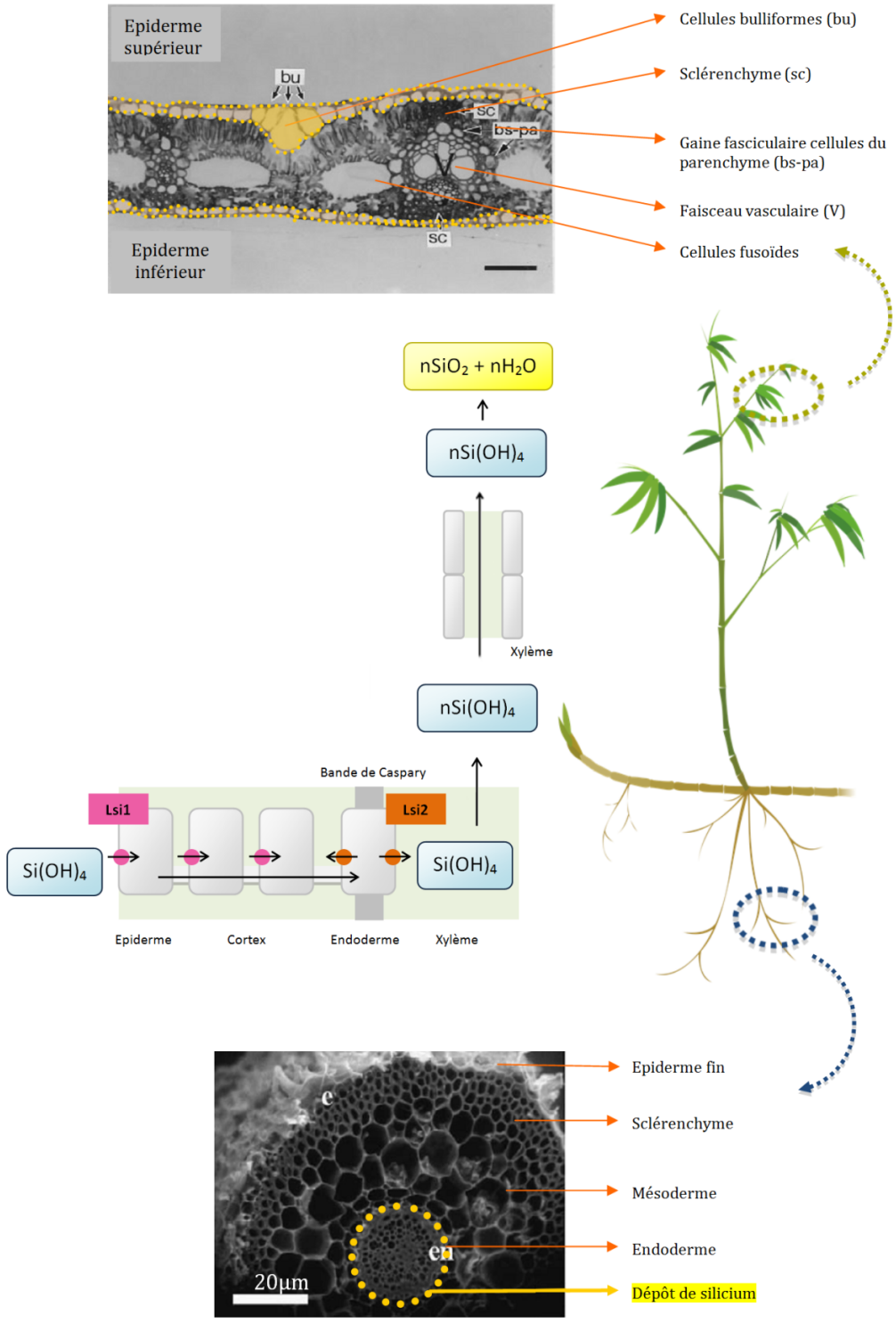


Figure I.11 **Absorption et localisation de Si dans le bambou. Le mécanisme d'absorption est celui qui est décrit pour le blé et le maïs (Mitani et al. 2009a; Mitani et al. 2009b). La localisation de Si a été schématisée sur les photographies MEB de feuilles (B) et de racines (C) de bambou selon les analyses EDX des études de Motomura et al (2006) (B) et de Lux et al (2003) (C).**

Le silicium doit ensuite être exporté du xylème vers les feuilles. Il est observé dans de nombreuses études que Si précipite majoritairement au niveau des sites d'évaporation. Sa répartition au sein de la feuille est alors la résultante d'un phénomène passif (Epstein 1999; Raven 2003). Dans le bambou, Ding et al. (2008b) mesurent une accumulation plus forte dans les feuilles, puis les branches, puis les tiges. C'est une tendance générale que l'on retrouve en comparant les moyennes des concentrations en Si données dans les différentes études : feuilles 94 $mg.g^{-1}$ > branches 17 $mg.g^{-1}$ > tiges 8 $mg.g^{-1}$ SiO_2 (Table I.1). Cette répartition peut indiquer le rôle de la transpiration et de l'évaporation pour le transport du silicium dans le bambou. Ding et al. (2008b) confirment également cela par une distribution des valeurs de $\delta^{30}Si$ qui sont plus élevées dans les parties de la plante où a lieu la transpiration, comme cela a été décrit pour le riz et la banane (Ding et al. 2005; Ding et al. 2008b; Opfergelt et al. 2005). Plusieurs études de Motomura et al (2000; 2004; 2006; 2008; 2002) ont été menées pour localiser le silicium dans les feuilles de bambou et comprendre si l'accumulation de Si est liée à un mécanisme passif ou actif. Au sein de la feuille, le silicium s'accumule majoritairement dans les cellules de l'épiderme, notamment des cellules bulliformes et des poils bicellulaires (Lux et al. 2003; Motomura et al. 2000). Or si l'accumulation de Si était uniquement le résultat de la transpiration, la précipitation de Si devrait être observée dans les cellules du chlorenchyme car beaucoup d'eau est évaporée via les stomates. Ces auteurs ont également étudié l'accumulation de Si dans les feuilles de *Phyllostachys pubescens et P. Bambusoides* durant leur durée de vie. Les feuilles accumulent Si pendant la phase de croissance mais aussi après leur maturation (Figure I.12). L'accumulation de Si est plus forte au printemps et en été, et diminue en hiver. Cette observation est expliquée par les variations de l'activité physiologique du bambou, en fonction des saisons. En été, la perte d'eau est plus forte, les stomates ouverts et la photosynthèse plus active (Motomura, 2008). Ce « schéma d'accumulation » est réitéré pendant la durée de vie des feuilles, qui est de trois ans pour l'espèce considérée. Ces résultats montrent que l'accumulation de silicium provient surtout de l'activité physiologique (évapo-transpiration, photosynthèse, etc.) et non pas des processus de développement et de croissance de la plante.

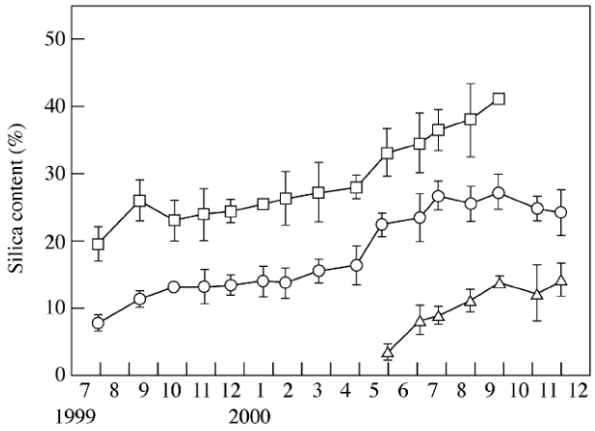


Figure I.12 Teneurs en SiO_2 dans des feuilles de bambous pendant trois ans : 1998 (carrés), 1999 (cercles), 2000 (triangles) (Motomura, 2002)

Ainsi, il semble que dans le bambou, on ne puisse expliquer le transport et l'accumulation de Si dans les parties aériennes uniquement par un transport passif. De plus un transporteur (Lsi 6), permettant d'exporter Si du xylème vers certaines cellules accumulatrices de Si de la feuille (ex cellule bulliforme), a été mis en évidence dans le riz (Yamaji et al. 2008). Ainsi la présence de ce transporteur indique que le dépôt de Si dans les cellules spécialisées est dépendant d'un transport symplasmique plutôt que d'un transport apoplasmique (Yamaji et al. 2008).

2.4. Fonction du silicium dans le bambou

Ma et Takahashi (2002) montrent que la quantité de silice des sols influe sur la quantité de silice concentrée dans les feuilles et le développement des bambousaies. Ils observent que les bambousaies ayant une forte productivité se développent sur des sols riches en silice biodisponible et se caractérisent par des teneurs élevées en silicium dans les feuilles (voir Table I.2). A l'inverse, les feuilles des bambousaies atteintes de maladie ou celles qui sont caractérisées par une productivité faible, ont des teneurs plus faibles en Si.

Table I.2 **Relation entre la teneur de SiO_2 des feuilles de bambou et la productivité de la bambousaie (Ma et Takahashi, 2002).**

	Bamboo species	
	Phyllostachys edulis	*Phyllostachys reticula*
	SiO_2 ($mg.g^{-1}$)	SiO_2 ($mg.g^{-1}$)
Good grove	79,9±10	105,5±9
Middel grove	60,7±13	81,2±10
Poor grove	50,4±6	70,9±8
Injured grove	53,8±6	60,9 ±9

Les expériences faites sur des bambousaies montrent également des effets remarquables sur la croissance avec une augmentation notable de la biomasse des feuilles, du nombre de bourgeons et de la rigidité des chaumes (Ma et Takahashi, 2002). Cependant, l'application de scories riches en silice dans différentes plantations de bambous en Chine ne s'accompagne pas d'une augmentation du rendement (Hong, 1994), mais au contraire d'une baisse dans certaines conditions expérimentales (absence d'engrais N, P et K). De plus, Motomura et al. (2008) montrent qu'à partir de teneurs en SiO_2 supérieures à 250 $mg.g^{-1}$, la photosynthèse peut être affectée par le dépôt de Si dans les cellules du chlorenchyme.

Malgré les très fortes quantités de Si accumulées dans le bambou, le rôle du silicium n'a été que très peu étudié. Pourtant de nombreux effets bénéfiques de Si sont décrits pour d'autre Poacées tel que le riz et le blé.

Les effets bénéfiques du silicium peuvent se répartir en trois types de fonctions :

- une fonction structurale, le silicium conférant une certaine résistance et rigidité des parois cellulaires ;
- une fonction protectrice, induisant une résistance aux pathogènes, aux insectes, aux champignons ;
- une fonction physiologique, en réduisant l'évapo-transpiration, en améliorant l'oxygénation des racines et les interactions avec d'autres éléments (dont les éléments traces métalliques).

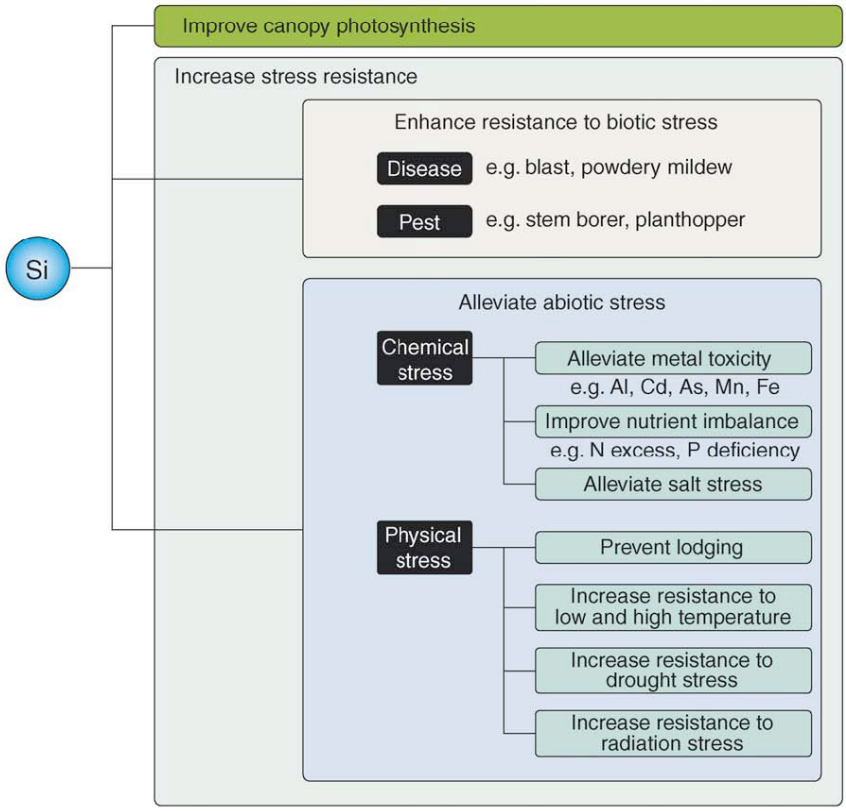


Figure I.13 Résumé des fonctions du silicium (schéma provenant de Ma and Yamaji 2006)

Les synthèses bibliographiques détaillant les effets positifs de Si sont nombreuses (Guntzer et al. 2011; Liang et al. 2007; Ma and Yamaji 2006) (Figure I.13). Nous allons présenter dans le paragraphe suivant uniquement le rôle du silicium face à une toxicité métallique.

2.5. Métaux et silicium

Plusieurs études testent l'efficacité d'un apport de silicium sur la toxicité de certains métaux : Al, Mn, Cd, Zn, Cu, As, B. Toutes les publications montrent qu'un apport de silicium diminue la toxicité des métaux, cependant les mécanismes d'action du silicium dans la plante restent peu connus. Les hypothèses proposées dans la littérature sont présentées ci-dessous. Dans la plupart des études, c'est la combinaison d'une ou de plusieurs hypothèses qui est observée pour expliquer le rôle de Si.

2.5.1 Modification des conditions environnementales

Dans des expérimentations sur sol, l'apport de Si (silicate de calcium, silicate de potassium) peut faire varier les propriétés physicochimiques du sol, en augmentant le pH par exemple,

entraînant ainsi une diminution de la phytodisponibilité des métaux (Chen et al. 2000; Gu et al. 2011; Liang et al. 2005; Treder and Cieslinski 2005; Zhao and Masaihiko 2007). L'apport de Si dans la solution de sol peut également modifier la spéciation des métaux et notamment former un complexe silicaté: Si-Al (Liang et al. 2001; Ma et al. 1997). Cependant cet effet indirect n'est pas suffisant pour expliquer la meilleure tolérance aux métaux, et est associé à un effet du Si *in planta*.

2.5.2 Augmentation de la biomasse

L'apport de silicium peut entraîner une augmentation la biomasse, diluant ainsi l'effet du polluant. Lors d'une expérience en pot, des ajouts de silicate de calcium (de 50 à 200 $mg.kg^{-1}$) dans du sol enrichi en Zn (100 $mg.kg^{-1}$) et en Cd (10 $mg.kg^{-1}$) ont provoqué une augmentation de biomasse chez le maïs (da Cunha and do Nascimento 2009). Zhang et al (2008) ont observé une augmentation significative de la biomasse de racines et de feuilles de riz cultivé en hydroponie à la suite d'un apport de 2 mM Si en solution, que ce soit sans ajout de Cd ou avec ajout de Cd en solution. De même Si améliore la croissance de plants de maïs (augmentation de la surface foliaire, du poids frais) (Vaculik et al. 2009), et de pak-choï, une brassicacée (variété de chou chinois) sans toxicité métallique (Song et al. 2009). Cependant, en condition environnementale non stressante, l'augmentation de la biomasse n'est pas systématique (Hammond et al. 1995; Oliva et al. 2011; Richmond and Sussman 2003).

2.5.3 Diminution de l'absorption des métaux

Un des principaux effets du Si vis-à-vis de la diminution d'une toxicité métallique est une réduction de la concentration des métaux dans les parties aériennes des plantes. Cela a été observé pour Cd, Zn, Mn, Cu dans des monocotylédones comme le riz (Shi et al. 2005b; Zhang et al. 2008), le maïs (Liang et al. 2005), mais aussi des espèces dicotylédones comme le pak-choï (Song et al. 2009), le concombre (Feng et al. 2009), l'arachide (Shi et al. 2010), *Erica Andevalensis* (Oliva et al. 2011). Cette diminution peut être expliquée par la barrière physique que forment les dépôts de Si sur les parois de l'endoderme entraînant une diminution de la porosité des parois cellulaires, et réduisant ainsi le passage des métaux dans le xylème (da Cunha and do Nascimento 2009). Shi et al. (2005b) ont également montré la réduction du flux apoplastique induit par Si lors d'une toxicité au Cd dans le riz, en mesurant la fluorescence d'un traceur apoplastique (PTS) qui renseigne sur l'efficacité du transport apoplastique. Il est cependant peu probable que le mécanisme réduisant la concentration des métaux dans les parties aériennes soit commun pour toutes les espèces étudiées et s'explique uniquement par une réduction du flux de métaux entrant. De plus, dans certaines études, Si entraine une

augmentation des teneurs en métaux dans la plante tout en réduisant leur toxicité (da Cunha and do Nascimento 2009; Vaculik et al. 2009).

2.5.4 Modification de la répartition des métaux

Plusieurs études ont observé la localisation des métaux et du silicium dans les tissus afin de comprendre leurs interactions. Da Cunha and do Nascimento (2009) ont localisé, par analyse optique histo-chimique, Cd , Zn et Si dans les mêmes cellules, au niveau de l'épiderme foliaire et au niveau de l'endoderme d'une racine de maïs. Zhang et al. (2008) ont observé par MEB-EDX une répartition similaire de Cd et Si dans une cellule bulliforme de feuille de riz. Ces auteurs proposent alors une co-précipitation de Si avec Cd ou Zn. Grâce à une observation par spectrométrie de perte d'énergie des électrons (EELS) Neumann and zur Nieden (2001) ont déterminé la composition des dépôts de silice dans *Cardaminopsis*. Ces auteurs suggèrent que le silicium intracellulaire forme des complexes Zn-silicate dans le cytoplasme des feuilles de *Cardaminopsis.* Ces silicates seraient ensuite transformés en $Si0_2$ dans le cytoplasme alors que les métaux seraient séquestrés dans la vacuole.

De nombreuses études analysent Si présent dans les différents compartiments en faisant des extractions chimiques : fluide apoplastique, fluide symplastique, parois cellulaires. Les premières études de ce type ont porté sur l'effet de Si face à une toxicité au Mn. Si réduit le développement de tâches brunes sur les feuilles, qui est un symptôme typique d'une toxicité à Mn. Pourtant les teneurs mesurées dans les plantes ne sont pas toujours diminuées après apport de Si, mais c'est la distribution du Mn dans une feuille qui est modifiée. Après un apport de Si dans le haricot et la vigne, Mn est réparti de manière homogène dans la feuille, ce qui expliquerait la réduction de sa toxicité (Horst and Marschner 1978; Iwasaki and Matsumura 1999). Les mécanismes d'action de Si entraînent donc une meilleure tolérance à Mn dans la cellule plutôt qu'un mécanisme d'exclusion (Rogalla and Romheld 2002b). Dans le concombre, l'apport de Si augmente la quantité de Mn lié aux parois cellulaires (90 %) et diminue la proportion symplasmique (10 %). Il est donc proposé par les auteurs que Si favorise la liaison de Mn aux parois cellulaires ce qui permet de diminuer la quantité de Mn du cytosol et donc sa toxicité (Rogalla and Romheld 2002a; b; Wang et al. 2000). Dans le potiron (*Cucurbita moschata Duch.*), 1,44 mM Si diminue la toxicité de Mn par une accumulation de Mn et Si sous une forme métabolique inactive autour de la base des trichomes sur la surface des feuilles (Iwasaki and Matsumura, 1999). Dans des feuilles de Cowpea, Iwasaki et al. (2002) observent une diminution de la quantité de Mn dans le fluide apoplastique combiné à un plus fort taux de Mn adsorbé sur les parois cellulaires après un apport de Si de 50 µM.

Cette modification de distribution a été observée pour d'autres métaux. Lors d'une étude en hydroponie, la concentration totale en Al des racines de maïs n'est pas influencée par Si, en revanche la partie échangeable de Al présent sur les parois cellulaires est modifiée (Wang et al. 2004). La partie facilement échangeable est réduite avec l'apport de Si et la répartition de Al dans les tissus des racines est modifiée. Ces auteurs proposent la formation de complexes biologiquement inactifs d'hydroxyaluminosilicate (HAS) dans l'apex des racines. Dans *Arabidopsis Thaliana*, Li et al. (2008) expliquent la meilleure tolérance à Cu par une séquestration de Cu dans les parois cellulaires. Leur étude montre également que la concentration en Cu des plantes n'est pas modifiée mais que la plante modifie sa stratégie de tolérance à Cu.

Cependant, ces résultats sont en désaccord avec ceux de Shi et al. (2010) dans *Arachis hypogaea* (arachide). Ces auteurs montrent que Si affecte la distribution de Cd dans les feuilles en diminuant la fraction du Cd liée aux parois cellulaires. Cette différence peut être expliquée par le comportement de Si qui diffère entre des espèces accumulatrices de Si comme le riz, et une espèce non accumulatrice : l'arachide.

2.5.5 Modification de l'expression de gènes

Un mécanisme qui semble être responsable d'une meilleure tolérance aux métaux est la stimulation du système anti-oxydant. Cet effet a été mis en évidence par la mesure de l'activité d'enzymes anti-oxydantes (par exemple SOD, POD, CAT, APX) dans *Arabidopsis* lors d'une toxicité à Cu (Khandekar and Leisner 2011), dans *Brassica chinensis* (pak-choï) et *Arachis hypogaea* (Arachide) lors d'une toxicité à Cd (Shi et al. 2010; Song et al. 2009), dans *Ducumis sativus* en réponse à une toxicité à Mn (Shi et al. 2005a), et dans le blé, la tomate et les épinards en réponse à une toxicité à B (Gunes et al. 2007a; Gunes et al. 2007b).

Récemment, Khandekar and Leisner (2011) ont montré qu'un apport de1,5 mM de Si, lors d'une toxicité au Cu chez *Arabidopsis Thaliana,* maintient ou augmente l'expression des gènes responsables de la production de métallothioneines (MTs) qui peuvent être des molécules chélatantes de Cu (Cobbett and Goldsbrough 2002).

Il semblerait également que Si ait un rôle sur l'activité des gènes PAL (Phenylalanine Ammonia-Lyase), diminuant leur expression lors d'un stress à Cu (Li et al. 2008). Cependant leur rôle dans la diminution de la toxicité n'est pas bien compris (Khandekar and Leisner 2011).

3. BILAN DU CHAPITRE I

Dans les racines, une partie importante de **Cu est immobilisée dans les parois cellulaires**. Le cuivre semble majoritairement absorbé sous forme réduite **Cu(I)** par le transporteur COPT1. Dans la cellule, mais aussi dans les vaisseaux conducteurs, Cu peut être chélaté par de **nombreux ligands**, comme les acides organiques, les métallochaperonnes, les phytochélatines, les glutathions, les métallothionéines. Dans la plante, le cuivre est majoritairement accumulé dans les racines.

Plusieurs **mécanismes de tolérance** sont proposés pour réduire une toxicité de Cu :

- réduction de l'absorption,
- séquestration de Cu dans les racines,
- séquestration du Cu dans la partie apoplasmique (parois cellulaires, vacuoles),
- complexation de Cu sous une forme moins toxique (phytochélatines, métallothionéines, acides organiques).

Le bambou est une **plante accumulatrice de silicium**. Si s'accumule majoritairement dans les feuilles où il peut atteindre une concentration de 410 $mg.g^{-1}$ $Si0_2$. Son absorption et son transport semblent être contrôlés par des mécanismes actifs et passifs.

Plusieurs études ont montré que **le silicium diminue la toxicité des ETM**. Dans la plante, les principaux mécanismes d'action observés sont les suivants :

- augmentation de la biomasse, diluant l'effet du polluant,
- amélioration de la capacité de la plante à séquestrer les métaux dans les racines,
- modification de la répartition des métaux entre la partie symplastique et apoplastique,
- modification de l'expression de certains gènes.

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CHAPITRE II

DISTRIBUTION AND VARIABILITY OF SILICON, COPPER AND ZINC IN DIFFERENT BAMBOO SPECIES

TABLE DES MATIERES

CHAPITRE II

DISTRIBUTION AND VARIABILITY OF SILICON, COPPER AND ZINC IN DIFFERENT BAMBOO SPECIES

Nous avons vu dans le premier chapitre que le bambou possède la capacité d'accumuler de fortes quantités de silicium dans ses tissus, mais les quantités accumulées varient très fortement d'une étude à l'autre. A titre d'exemple, Li et al. (2006) mesurent une concentration moyenne de 31 $mg.g^{-1}$ dans les feuilles de bambou *Phyllostachys heterocycla* var. *pubescens* alors que Ding et al. (2008) reportent une concentration de 90,4 $mg.g^{-1}$ en moyenne dans différentes espèces. Nous nous sommes donc interrogés sur l'origine de cette variabilité : tient-elle aux paramètres environnementaux contrastés entre les études précitées ou bien à l'impact des différentes espèces étudiées ? Sachant qu'il existe plus de 1200 espèces, appartenant à plus de 70 genres (Kleinhenz and Midmore 2001), il apparaît important de déterminer si l'accumulation de Si est influencée par l'espèce de bambou. Deux types de bambous sont distingués selon la morphologie de leurs rhizomes (Figure II.0)(McClure 1966) :

- les bambous monopodiaux (ou traçants) ayant des rhizomes « leptomorphes »,
- les bambous sympodiaux (ou cespiteux) ayant des rhizomes « pachymorphes ».

Les entrenœuds des rhizomes leptomorphes sont longs et minces, alors que ceux des rhizomes pachymorphes sont courts et épais. Les différences d'anatomie du rhizome peuvent être considérées comme une adaptation aux conditions climatiques de la zone d'origine des bambous. Les bambous monopodiaux sont originaires de régions marquées par un climat tempéré avec des hivers froids et humides, alors que les bambous sympodiaux sont originaires de régions à climat tropical avec une saison sèche marquée. Grâce à des entrenœuds plus courts, le rhizome pachymorphe est moins sensible à la sècheresse. Contrairement aux bambous

monopodiaux qui peuvent coloniser une surface très importante, les bambous sympodiaux ne s'étendent que très lentement et leurs chaumes demeurent serrés en une touffe très dense.

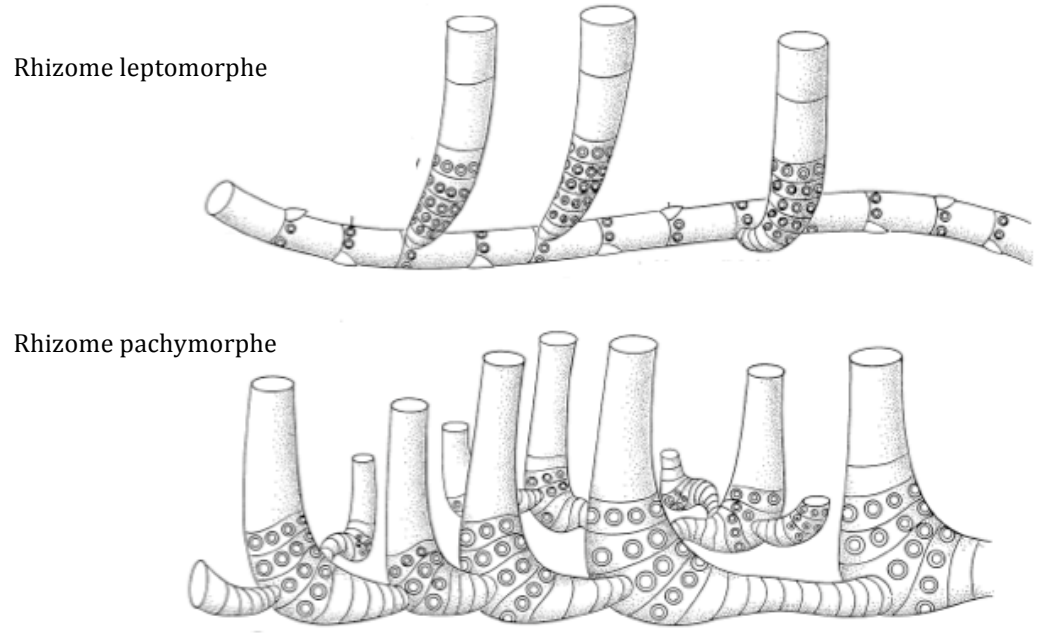

Figure II.0 Types de rhizomes, figure modifiée d'après (Soderstrom and Young 1983)

Ainsi, le nombre important d'espèces de bambous et la distinction entre deux types majeurs nous ont amenée à nous interroger sur l'influence des espèces sur les concentrations en Si mais aussi en Cu et Zn. Les objectifs de ce chapitre sont de :

1) déterminer la variabilité des concentrations de Cu, Zn et Si en comparant 16 espèces de bambous se développant dans les mêmes conditions environnementales, sur un sol volcanique de l'île de la Réunion,
2) obtenir des concentrations de références pour Si, Cu et Zn dans les parties aériennes des bambous,
3) identifier d'éventuelles corrélations entre les concentrations de Si, Cu et Zn dans les bambous.

L'article présenté dans ce chapitre intitulé "Distribution and variability of silicon, copper and zinc in different bamboo species" décrit les variations de concentrations de Si, Cu, Zn dans les différents tissus des bambous et entre les espèces, en discutant des mécanismes impliqués au regard des connaissances issues de la bibliographie.

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Distribution and variability of silicon, copper and zinc in different bamboo species

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ABSTRACT

With a high growth rate and biomass production, bamboos are frequently used for industrial applications and recently have proven to be useful for wastewater treatment. Bamboos are considered as Si accumulators and there is increasing evidence that silicon may alleviate abiotic stresses such as metal toxicity. The aim of this study was to investigate the extent of metal concentrations and possible correlations with Si concentrations in plants. This study presents, for the first time, reference values for silicon (Si), copper (Cu) and zinc (Zn) concentrations in stems and leaves of various bamboo species grown under the natural pedo-climatic conditions of the island of Réunion (Indian Ocean). A broad range of silicon concentrations, from 0 (inferior to detection limit) to 183 mg g^{-1} dry matter (DM), were found in stems and leaves. Mean leaf Cu and Zn concentrations were low, i.e. 5.1 mg kg^{-1} DM and 15.7 mg kg^{-1} DM, respectively. Silicon, Cu and Zn concentrations increased over the following gradient: stem base < stem tip < leaves. Significant differences in Si, Cu and Zn contents (except Zn in the stem) were noted between bamboo species, particularly between monopodial and sympodial bamboo species, which differ

in their rhizome morphology. Sympodial bamboos accumulated more Si and Cu than monopodial bamboos, in both stems and leaves, whereas sympodial bamboos accumulated less Zn in leaves than monopodial bamboos. The findings of this study suggest that a genotypic character may be responsible for Si, Cu and Zn accumulation in bamboo.

Keywords: trace element, silica, Poaceae, genotypic variability, island of Réunion

1. INTRODUCTION

Bamboos are widespread plants belonging to the grass family (Poaceae). They are commonly found in temperate, tropical and subtropical regions and widely used for industrial purposes, as fresh edible shoots, making paper, building material and even in medicines. Bamboos are known for their resistance to a wide range of stress factors and their high growth rate and biomass production, with potential uses in phytoremediation. The PHYTOREM company (France) has developed BAMBOO-ASSAINISSEMENT® technology for wastewater treatment (Arfi et al. 2009). The company is currently optimizing the phytoremediation capacity of bamboos under tropical climatic conditions.

The uptake and accumulation of essential nutrients such as N, P and K are well documented in bamboos (Embaye et al. 2005; Shanmughavel and Francis 2001), but metal accumulation has been poorly documented so far. Metals are naturally present in the pedo-geochemical background of soils at various levels and many metals are essential to plants, but they may be toxic at higher concentrations. Metals accumulate in soil due to anthropogenic contamination through fertilizer and organic manure applications, industrial and municipal wastes, irrigation, and wet and/or dry deposits (Doelsch et al. 2010; Novak J et al. 2004). Phytoremediation techniques have been put forward as alternatives to remediate metal contaminated soils, especially agricultural soils (McCutcheon and Schnoor 2003). One of the limitations of such technologies is the availability of plant species adapted to specific environmental conditions and, accumulating and/or tolerant to large metal or metalloid concentrations in soils (Keller 2005). Bamboos are potentially good candidates for phytoremediation because of their widespread distribution, their easy and well-known propagation mode, the broad range of species and their possible additional use as raw material. To our knowledge, there is no data available on metal concentrations in bamboos, especially under natural conditions. In this study, we focused on the case of copper (Cu) and zinc (Zn), two metals which may be present at high concentration in wastewater.

Like many Poaceae species (sugarcane, rice, wheat, etc.), bamboos are considered as Si accumulators, with Si concentrations ranging from 3 to 410 mg g^{-1} SiO_2 (DM) (Ding et al. 2008; Li et al. 2006). The variability in Si content in bamboo may be explained by: (i) the available pool of Si in soil (Henriet et al. 2008; Jones et al. 1967); (ii) the increase in Si content during bamboo ageing (Motomura et al. 2002) and (iii) genetic variability among species. This last point has

never been studied in bamboos, whereas it may be important because of the high number of bamboo species: a total of about 1030 bamboo species (77 genera) are grouped in the sub-family Bambusoideae, within the family Poaceae. In order to clarify the role of the species in the Si uptake capacity, data are thus needed on Si concentrations in bamboos of the same age and grown in similar soils.

In rice or wheat, there is increasing evidence that silicon may alleviate metal toxicity (Liang et al. 2007). Several mechanisms have been proposed to explain the role of Si in metal tolerance, such as limitation of metal uptake, reduction of root-to-shoot translocation or changes in metal allocation within the plant. In bamboos, the role of Si on metal tolerance has not been investigated. The first step before any attempt to test bamboos in contaminated soils for phytoremediation technologies is to investigate, under natural conditions, the extent of metal concentrations and possible correlations with Si concentrations in plants.

The objectives of the present study were to: (1) determine the variability in Cu and Zn (for the first time) and Si concentrations between species by comparing 16 bamboo species grown under similar environmental conditions, i.e. a volcanic soil on the island of Réunion (Indian Ocean, France), (2) obtain reference values for Si, Cu, Zn concentrations in the different above-ground parts of bamboos and, (3) highlight the possible relationship between Si, Zn and Cu in bamboos growing in a natural soil under tropical climatic conditions.

2. MATERIALS AND METHODS

2.1. Geographical area of the study, soil description and sampling procedure

Bamboo samples were collected from a 3.5-ha bamboo nursery in Réunion (Indian Ocean island), France (Mr Perrussot, Le Guillaume, Saint Paul). 130 different species have been maintained in this nursery since 1987. The climate is both tropical and oceanic, with easterly prevailing winds. The studied site (21°03'51 S, 55°19'28 E) is on the west side of the Piton des Neiges shield volcano, 1035 m above sea level. The mean annual precipitation is 1700 mm, and the temperature ranges from 10 to 28°C, with hot and humid summers and warm and wet winters. Soil in this area is classified as a chromic Andosol developed from Piton des Neiges volcanic material (Raunet 1991).

Sixteen bamboo species belonging to six genera were sampled in the same field in October 2008. These bamboos were selected because they may be good candidates for phytoremediation under tropical conditions, due to their high biomass production and good adaptations to tropical climates. Bamboo species differ the rhizome form, i.e. the underground part from which roots and shoots grow from nodes. According to the rhizome morphology, bamboos are divided into monopodial bamboos with leptomorph rhizome systems, and sympodial bamboos with pachymorph rhizome systems (McClure 1966). These differences in rhizome systems can be regarded as adaptations to the climatic conditions from which the bamboos originated: monopodial bamboos are native to temperate climates and sympodial bamboos are native to tropical climates (Kleinhenz and Midmore 2001). Ten out of the 16 species were monopodial bamboos, i.e. *Dendrocalamus giganteus, Dendrocalamus strictus, Bambusa bambos, Bambusa oldhamii, Bambusa vulgaris* 'Vittata', *Bambusa multiplex* 'Golden Goddess', *Bambusa multiplex* 'Alphonse Karr', *Bambusa tuldoides, Gigantochloa* sp. 'Malay Dwarf' and *Thyrsostachys siamensis*; and six were sympodial bamboos, i.e. *Phyllostachys aurea, Phyllostachys bambusoïdes* 'Castillon', *Phyllostachys bissetii, Phyllostachys flexuosa, Phyllostachys humilis* and *Pseudosasa japonica.*

For each species, three different 1-year-old bamboo specimens were selected to sample stems and leaves. Two samples were taken from each stem: one at the third internode above the soil surface, further referred to as the "stem base", and the second at the tip of the stem, further referred to as the "stem tip". For each stem, one bulk leaf sample was taken. A total of 94 stem samples and 47 leaf samples were collected.

2.2. Soil material and analysis

Soil was sampled under at least one bamboo of each species. A total of 18 soil samples were obtained by collecting topsoils (0-25 cm) with a gauge auger and mixing five replicates for each sample. Only steel or plastic tools (knife, spade and shovel) were used for sampling in order to avoid heavy metal contamination.

Soil samples were air-dried, crushed and passed through a 2-mm sieve before analysis. pH_{water} (soil/water ratio=1:5) was measured according to ISO 10390.

For Si and trace element analyses, a representative soil subsample was ground to 100 µm particle size before digestion. For Cu and Zn analysis, calcination at 450°C was followed by total dissolution performed using a mixture of HF, HNO_3 and $HClO_4$ (ISO 14869-1). For SiO_2 analysis, complete dissolution was obtained by alkaline fusion of the soil sample in the presence of sodium peroxide (AFNOR standard BP X 30-428).

Phytoavailable fractions of Cu and Zn were estimated using an extraction method (Collin and Doelsch 2010). After shaking 50 ml of 1 mol L^{-1} NH_4NO_3 solution and 20 g dry soil sample for 2 h at 30 rpm in a room at 20 ± 2°C, the extracts were centrifuged at 1000 g for 15 min. The supernatant was filtered through a membrane unit filter (0.22 µm).

All extracts were acidified with HNO_3 and stored in polyethylene bottles at 4°C before analysis. All reagents were analytical grade and only ultrapure water (Purelab Prima plus Classic from Elga Labwater) was used. All glass and plastic ware used for the experiments was previously soaked overnight in nitric acid and rinsed with ultrapure water. Three replicates were performed for each sample. Blank tubes (containing reagent but no soil) were also taken throughout each procedure.

Silicon and trace element concentrations were then determined with an inductively coupled plasma-optical emission spectrometer (ICP–OES Vista-PRO, Varian, Inc.) with an axially viewed plasma system and a charge coupled device detector. For quality control, in-house reference samples and certified samples (CRM 7001 Light Sandy Soil and CRM 7004 Loam, Analytica) were used every 10 samples and each measurement was conducted in duplicate. The detection limits were 0.025 $mg.kg^{-1}$ for Cu and Zn. The measurement uncertainty was less than 10 %.

2.3. Plant material and analysis

Stems and leaves were washed with distilled water and dried at 60°C until constant weight. They were subsequently mixed, ground and homogenised. Sub-samples were dried at 80°C until constant weight to determine the dry weight.

The plant samples underwent dry mineralisation for Zn and Cu trace element analyses. During mineralisation, the Si content was determined by gravimetric quantification: 500 mg of dried plant material was placed in a platinum dish and gradually heated to 500°C. Silica was eliminated in the ash with HF. The Si weight was determined after cooling. The ash was dissolved in HCl and the Cu and Zn contents in the solutions were analysed by ICP-OES. For quality control, in-house reference samples and certified samples (Astrasol-Mix, Analytika) were used every 20 samples and each analysis was conducted in duplicate. The measurement uncertainty was ± 15 %. The quantification limit for Si was 5 mg g^{-1}.

Copper and Zn concentrations are expressed as mg kg^{-1} of dry matter (DM) and Si concentrations are expressed in mg g^{-1} DM SiO_2 in order to have results directly comparable with the literature.

2.4. Statistical analyses

The Minitab 15.1 software package (Minitab, Inc.) was used for statistical analyses. Mean concentrations at the stem base, the stem tip and leaves were compared using a paired t-test at the 95 % confidence level.

For each plant part, concentrations in the different species were analysed by ANOVA. We used one-way ANOVA at the 95 % confidence level with bamboo “species” (16 levels) as the main factor, followed by Tukey’s post hoc test at the 95 % confidence level to evaluate differences in Cu, Si and Zn concentration at the stem bases, the stem tips and the leaves. Differences between monopodial and sympodial bamboos were analysed using a general linear model with the type of bamboo as factor (2 levels). Mean concentrations from both types (sympodial or monopodial) were then compared using Tukey’s post hoc test at the 95 % confidence level.

3. RESULTS

3.1. Soil

Total Si, Cu and Zn soil concentrations are presented in Table II.1. The mean total concentrations in the soil samples were 213 ± 23.3 mg g^{-1} for SiO_2, 26.5 ± 5.8 mg kg^{-1} for Cu and 113 ± 18.2 mg kg^{-1} for Zn, and the average soil pH was 6.1 ± 0.3. Within the study framework, soil variations in Si, Cu, Zn concentration were reduced, while the coefficient of variation of concentrations within the sampled area was of 11 % for Si and 21.8 % for Cu and 16.2 % (Table II.1). The Cu NH_4NO_3-extractable fractions (Cu_{NH4NO3}) were below the detection limit in all soil samples and the mean Zn NH_4NO_3-extractable fractions (Zn_{NH4NO3}) were 0.3 ± 0.23 mg kg^{-1} (Table II.1).

Table II.1 Characteristics of the total soil population (N=18)

	pH_{water}	SiO_2	Cu total	Zn total	Cu_{NH4NO3}	Zn_{NH4NO3}
		mg g^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
Mean	6.1	213	26.5	113	<0.025	0.30
Median	6.0	208	28.2	114	<0.025	0.25
Standard deviation	0.3	23.3	5.8	18.2		0.23
Coefficient of variation	5%	11%	21.8 %	16.2 %		
Minimum	5.5	175	13.2	80.2		
Maximum	6.5	254	34.1	142		
Mean of Reunion soils[a]		216	58.2	162	0.02	0.32
Mean of world soils[b]			20	63		

[a] Data from Doelsch et al. (2006) and Collin and Doelsch (2010)
[b] Data from Kabata-Pendias and Mukherjee, (2007)

3.2. Between plant parts

Table II.2 shows the average Si, Cu, Zn concentrations at the stem base, at the stem tip and in the leaves. Within the stem, Si, Cu and Zn concentrations significantly increased from the stem base to the tip. The mean concentrations at the stem base were under quantification limit for Si, 3.5 mg Cu kg^{-1} and 7.0 mg Zn kg^{-1}, whereas at the stem tip, the mean concentrations were 21 mg Si g^{-1}, 4.5 mg Cu kg^{-1} and 14.8 mg Zn kg^{-1}. The Si content at the stem tip was thus more than 4.1-fold higher than at the stem base, and this difference was greater than that noted for Cu and Zn: 1.3- and 2.1-fold, respectively. The leaf Si and Cu concentrations, i.e. 109 mg g^{-1} and 5.1 mg kg^{-1} respectively, were significantly higher than in the stem.

No correlations between Cu, Zn and Si contents were found in stems and leaves (R^2<0.18). There was no longer any correlation between the total Si, Cu and Zn soil concentration and the Si, Cu and Zn plant concentration (R^2<0.2), or between the Zn_{NH4NO3} fraction and the Zn plant concentration (R^2<0.3) (for Cu, Cu_{NH4NO3} fractions are below the detection limit).

Table II.2 **SiO2, Cu and Zn concentrations in the different plant parts (N=47). Data are expressed as mg kg^{-1} of dry matter for Cu and Zn concentrations and mg g^{-1} of dry matter for SiO_2 concentrations.**

		Stem bases	Stem tips	Leaves
	Number of samples	*47*	*45*	*47*
SiO_2	Mean ± standard deviation	<QL$^{\tau}$	21$^{a\tau\tau}$ ±16	109^{b} ±30
	Coefficient of variation	<QL	785	279
	Range	5 - 12	5 - 102	23 - 183
	ANOVA $F_{SPECIES}$	-	2.12*	2.23*
Cu	Mean ± standard deviation	3.5^{a}±1.0	4.5^{b} ±2.0	5.1^{c} ±1.0
	Coefficient of variation	28.1	43.9	18.8
	Range	1.8-6.4	1.7-10.9	3.4-7
	ANOVA $F_{SPECIES}$	4.99***	5.53***	6.8***
Zn	Mean ± standard deviation	7.0^{a}±6.3	14.8^{b} ±16	15.7^{b} ± 5.6
	Coefficient of variation	89.5	110	35.9
	Range	1.5-36.7	1.7-87.6	9.8-37.8
	ANOVA $F_{SPECIES}$	4.73***	1.56^{NS}	2.54*

$^{\tau}$<QL : under the quantification limit of 5 mg g^{-1}

$^{\tau\tau}$ Values followed by same letter within the same line are statistically not different according to Tukey test at the 95 % confidence level.

0ne-way analysis of variance and F-test for SiO_2, Cu, Zn, concentrations in stem bases, stem tips and leaves relative to the species factor. NS : not significant p>0.05 Sig.:* = P <0.05, *** P < 0.001

3.3. Between plant species

Mean Si concentrations were significantly different between species in leaves and in the stem tips (Table II.2). In our study, there was substantial variation in the Si content range between the 16 species: from 5.7 mg g^{-1} in *Phyllostachys aurea* to 56 mg g^{-1} in *Bambusa multiplex* 'Golden Goddess' at the stem tip, and from 82 mg g^{-1} in *Phyllostachys bissetii* to 159 mg g^{-1} in *Dendrocalamus strictus* in the leaves (Table II.3). We assumed that this wide Si content range could mainly be explained by the genotypic variation since both soil and climatic conditions were similar for all the sampled species.

In order to explain the differences in Si content between species, we first compared Si concentrations between the two genera *Phyllostachys* and *Bambusa* (data not shown), and the Si concentration was not significantly different. However, we found significant differences between sympodial and monopodial bamboos (Figure II.1), with the Si concentrations being significantly higher in sympodial than in monopodial bamboos, both in stems and leaves.

For Cu, differences between species were significant both in stems and leaves (Table II.2). *Bambusa multiplex* "Alphonse Kar" had the highest Cu concentration, with: 7.6 mg kg^{-1} in stem tips, while *Phyllostachys bambusoides* "Castillon" had the lowest concentration, with 2.0 mg kg^{-1} in stem tips, and 3.5 mg kg^{-1} in the leaves (Table II.3). Grouping species by types showed that Cu concentrations were significantly higher in sympodial than in monopodial bamboo species, both in stems and leaves (Figure II.1). The range of Zn concentrations in the leaves and at the stem base was broad and significantly different between species (Table II.2). For example, the Zn concentration in the stem bases ranged from 2.3 to 21.7 mg kg^{-1} in *Gigantochloa* sp. "Malay Dwarf" and *Thyrsostachys siamensis*, respectively (Table II.3) but the influence of species with respect to concentrations in the stem tips was not significant. Unlike Cu, Zn concentrations in leaves were significantly higher in monopodial than in sympodial bamboo species. In stems, Zn concentrations were not influenced by the type of bamboo (Figure II.1).

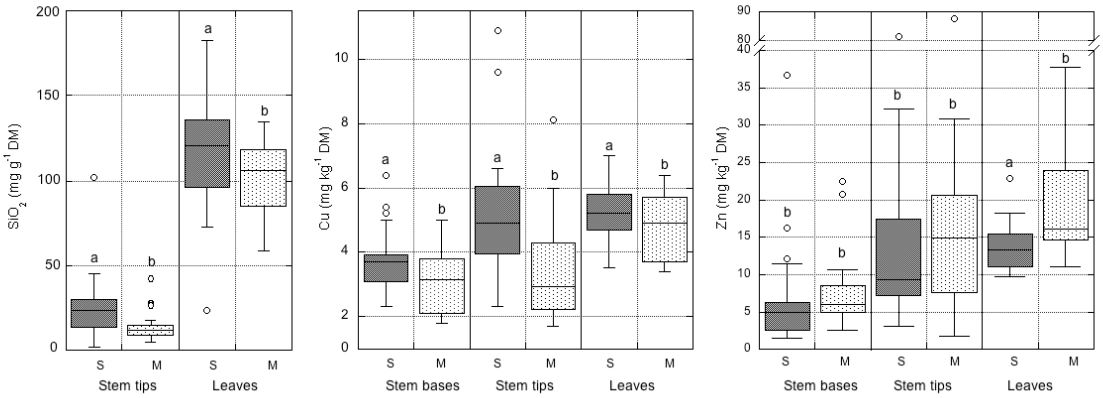

Figure II.1 **Comparison of SiO_2, Cu, Zn concentrations in stem bases, stem tips and leaves of monopodial bamboos (n= 18) and sympodial bamboos (n = 29). DM: dry matter; Boxes represent the median (vertical solid line) and 25–75 % percentile. Whiskers represent the 90th and 10th percentile. Significant differences were determined by a post hoc comparison of means (Tukey test after ANOVA; *P* < 0.05) and are indicated by different letters.**

Table II.3 Mean SiO_2, Cu and Zn concentrations in different plant parts in each species. For each species, the sample number is 3. The type of bamboo is indicated: S: Sympodial bamboo species, M: Monopodial bamboo species.

Si (mg.g $^{-1}$)			Cu (mg.kg $^{-1}$)			Zn (mg.kg $^{-1}$)				
Stem bases	Stem tips	Leaves	Stem bases	Stem tips	Leaves	Stem bases	Stem tips	Leaves	Type	Species
<5	5,7 ± 1,0	106 ± 1,6	2,3 ± 0,6	3,3 ± 0,8	5,9 ± 0,5	17,0 ± 8,0	41,0 ± 40,4	15,7 ± 1,3	M	*Phyllostachys aurea*
<5	18 ± 9,2	110 ± 0,8	2,5 ± 1,0	2,0 ± 0,3	3,5 ± 0,1	7,2 ± 3,0	11,1 ± 4,9	24,1 ± 3,8	M	*Phyllostachys bambusoïdes* 'Castillon'
<5	13 ± 4,3	82 ± 2,5	3,8 ± 0,4	6,5 ± 1,4	5,5 ± 0,6	5,0 ± 0,5	13,9 ± 6,6	26,0 ± 10,9	M	*Phyllostachys Bissetti*
<5	10 ± 2,8	84 ± 3,0	4,8 ± 0,2	3,8 ± 0,8	5,5 ± 0,7	8,9 ± 1,6	4,2 ± 3,1	14,3 ± 3,0	M	*Phyllostachys flexuosa*
<5	27 ± 15	96 ± 1,7	2,6 ± 0,5	3,3 ± 0,9	4,5 ± 0,6	6,0 ± 0,7	8,0 ± 2,6	14,5 ± 1,6	M	*Phyllostachys humilis*
<5	10 ± 3,9	113 ± 2,5	3,2 ± 0,4	2,2 ± 0,3	3,9 ± 0,3	3,3 ± 1,4	19,7 ± 3,8	19,5 ± 10,1	M	*Pseudosasa japonica*
<5	28 ± 0,6	140 ± 2,3	5,1 ± 1,8	4,2 ± 0,2	4,0 ± 0,7	6,3 ± 0,1	12,2 ± 0,2	15,8 ± 3,5	S	*Bambusa bambos*
<5	18 ± 8,1	97 ± 1,4	4,5 ± 0,9	7,6 ± 1,8	6,2 ± 0,2	4,8 ± 1,1	11,6 ± 5,7	13,8 ± 0,7	S	*Bambusa multiplex* 'Alfonse Kar'
5,5 ± 4,8	56 ± 42	121 ± 2,2	3,1 ± 0,3	7,4 ± 3,0	5,9 ± 0,4	2,4 ± 0,3	11,7 ± 7,9	13,4 ± 2,4	S	*Bambusa multiplex* 'golden godess'
<5	13 ± 16	115 ± 3,0	3,9 ± 0,3	4,6 ± 1,1	5,0 ± 0,4	6,0 ± 0,6	5,7 ± 2,8	13,2 ± 0,2	S	*Bambusa tuldoides*
<5	15 ± 4,5	75 ± 5,4	3,5 ± 0,5	5,9 ± 0,7	4,8 ± 0,8	5,3 ± 2,5	9,5 ± 5,6	16,5 ± 5,8	S	*Bambusa vulgaris* 'vittata'
<5	19 ± 15	95 ± 2,3	3,0 ± 0,4	4,1 ± 0,8	6,3 ± 1,0	4,1 ± 0,6	10,3 ± 5,8	15,8 ± 0,9	S	*Bambusoïdes oldhamii*
5,5 ± 3,4	26 ± 7,5	101 ± 2,6	3,7 ± 0,1	4,5 ± 0,2	5,8 ± 0,2	2,7 ± 1,4	6,2 ± 1,4	10,6 ± 1,2	S	*Dendrocalamus giganteus*
<5	17 ± 8,0	159 ± 2,3	4,1 ± 1,0	3,5 ± 1,4	4,5 ± 0,7	8,8 ± 3,5	9,1 ± 0,8	10,7 ± 0,5	S	*Dendrocalamus strictus*
5,9 ± 2,8	30 ± 6,3	135 ± 2,9	2,6 ± 0,3	3,2 ± 0,8	5,1 ± 0,2	2,3 ± 0,5	18,5 ± 2,4	12,9 ± 4,2	S	*Gigantocloa sp* « Malay Dwarf »,
6,4 ± 4,7	27 ± 5,4	124 ± 1,0	4,4 ± 0,7	4,9 ± 1,1	4,4 ± 0,6	21,7 ± 13,2	39,5 ± 36,5	14,6 ± 3,9	S	*Thyrostachys siamensis*

4. DISCUSSION

4.1. Phytoavailability of Si, Cu and Zn

The mean total SiO_2 concentration of the soil samples (213 ± 23.3 mg g^{-1}) was similar to the mean Si concentration previously measured in Réunion soils, i.e. 216 mg g^{-1} (Table II.1) (Doelsch et al. 2006). This volcanic soil contains easily weatherable silicate minerals due to the presence of poorly crystalline minerals and particularly very low polymerized aluminosilicates that may contribute the phytoavailable Si pool (Basile-Doelsch et al. 2005).

The mean total Cu concentration (Table II.1) was close to the mean concentration in world soil (20 mg kg^{-1}), but lower than the mean concentration measured in a set of Réunion soils (Doelsch et al. 2006). The mean Zn concentration was also much higher than the mean soil concentration given by Kabata-Pendias and Mukherjee (2007), and slightly lower than the mean concentration calculated for Réunion soils (Doelsch et al. 2006). These larger Cu and Zn concentrations as compared to world soil concentrations could be explained by the origin of the parent material: soils formed from the Piton des Neiges volcanic material are characterised by low Cr, Cu and Ni concentrations and relatively high Zn concentrations (Doelsch et al. 2006). Indeed, these latter authors demonstrated that the natural pedogeochemical background could account for the high Cr, Cu, Ni and Zn concentrations in Réunion soils. In spite of these higher average total concentrations, the Cu and Zn NH_4NO_3-extractable fractions were low, which is consistent with the findings of Collin and Doelsch (2010), who demonstrated the low phytoavailability of Cu, Cr, Ni and Zn in Réunion soils. The absence of correlations between Cu and Zn total concentration in soil and Cu, Zn concentration in plants (R^2<0.2) was thus not surprising and confirmed the low phytoavailability of these elements.

4.2. Origin of Si variation in bamboos

Silica concentrations in leaves were within the 20 to 410 mg g^{-1} Si range reported in several previous studies (Table II.4). Silicon concentrations in stems ranged from <5 to 102 mg g^{-1}, which is a broader range than reported in the literature presented in Table II.4 (3 – 44 mg g^{-1}). The mean Si concentration measured at the stem tip in the 16 species (21 mg g^{-1}) was significantly higher than the concentration at the stem base (under the detection limit), which is out of line with the findings of Li et al. (2006). Indeed, at the stem base in the Moso bamboo stand (*Phyllostachys heterocycla* var. *pubescens*), these authors measured a concentration of 44 mg g^{-1} Si, whereas they measured 1.5 mg g^{-1} Si in the stem. However, the exact locations of the analysed samples corresponding to "base of the stem" and the "stem" were not given by Li et al.

(2006), thus limiting the possibility of comparison with our results. Silicon concentrations thus varied between plant parts, with the accumulation of Si in leaves and a concentration gradient along the stem. To explain the distribution with the plant, transpiration has been proposed as the main mechanism for Si transportation and precipitation in Chinese bamboos (Ding et al. 2008). The evidence is based on the total silicon content and $\delta^{30}Si$ values, which both increase from the stem, through the branches to the leaves. The results of this study are consistent with the hypothesis of silica being carried passively through the transpiration stream and being deposited where water is lost in largest quantities, as proposed by Ding et al. (2008). However, in an Si accumulator, it is likely that active silicon distribution mechanisms in bamboo shoots are also required (Ma and Yamaji 2008).

In leaves and at the stem tips, there was marked variation in the Si content between the 16 sampled species (Table II.2). This result is in good agreement with the findings of Hodson et al., (2005) who showed that variability in Si content over 735 plant species is mainly explained by genotypic variation rather than environment. However, although differences in Si content between plant families are well known (Hodson et al. 2005), genotypic variation in Si concentration within species is less documented. The effect of genotypic variation has been studied in a few other plants, particularly in other Poaceae species. For example, in 52 sugarcane genotypes grown in the same soil, the shoot Si concentration ranged from 6 to 8 mg g^{-1} (Deren et al. 1993). In rice, the same authors compared the Si concentration in plants from 18 cultivars grown in greenhouse experiments and in fields amended with various amounts of Si. The silica concentration in plant tissues ranged from 3 to 60 mg g^{-1} depending on the Si supply, but within each Si treatment the coefficient of variation due to genotypic differences was 9 to 17 % (Deren et al. 2001; Deren et al. 1993). In field trials, Winslow et al. (1997) observed differences between *japonica* and *indica* rice cultivars, with mean Si concentrations in husks of 2 and 1.2 mg g^{-1}, respectively. In a survey of about 400 barley species, the Si concentration in grain ranged from 0 (under the detection limit) to 3.8 mg g^{-1} in hulled barley cultivars (Feng Ma et al. 2003). However, no significant differences in Si absorption were observed in three different banana genotypes grown in hydroponic culture conditions (Henriet et al. 2006). Although genotypic differences in Si content have been found in other Poaceae species such as rice, sugarcane and wheat, this is not systematic in all species and may question the genetic mechanisms that control Si accumulation in different species.

Table II.4 Inventory of Si data found in the literature.

	Mean ± standard deviation	min	max	Number of samples	References
Leaves	94 ± 75	20	410	22	1 to 6
Leaves of monopodial bamboos	82 ± 14	31	159	13	1-2-3-4-6
Leaves of sympodial bamboos	114 ± 118	20	410	9	1-5-6
Stems	8 ± 8	3	44	22	1-2-6
Stems of monopodial bamboos	5 ± 5	2	44	13	1-2-6
Stems of sympodial bamboos	12 ± 10	3	30	9	1-6
Branches	17 ± 4	8	20	8	1-6
Branches of monopodial bamboos	17 ± 5	8	20	7	1-6
Branches of sympodial bamboos	16			1	1
Rhizomes	7 ± 4	3	17	12	4-6
Rhizomes of monopodial bamboos	5 ± 2	3	9	5	6
Rhizomes of sympodial bamboos	8 ± 4	5	17	7	4-6
Roots	18 ± 22	6	73	9	1-6
Roots of monopodial bamboos	18 ± 23	6	73	8	1-6
Roots of sympodial bamboos	9			1	1

Data are expresses as SiO_2 mg g^{-1} of dry matter for Si concentrations

1: Ding et al. 2008; 2: Li et al. 2006; 3: Lux et al. 2003; 4: Meunier et al. 1999; 5: Motomura et al. 2002; 6: Ueda and Ueda. 1961 (from Ma and Takahashi, 2002).

Si concentrations were significantly higher in sympodial bamboos than in monopodial bamboos, in both stems and leaves (Figure II.1). This is consistent with findings in the literature (Table II.4). For example, higher Si concentrations were reported in sympodial bamboo leaves (114 mg g^{-1}) than in monopodial bamboos leaves (82 mg g^{-1}) (Table II.4).

There are marked morphological differences between pachymorph rhizomes (sympodial) and leptomorph rhizomes (monopodial), but no differences in root development and nutrient absorption capacity have been noted between the two types (Kleinhenz and Midmore 2001). Since these authors also reported that at least 80 % of the total root biomass is located in the topsoil (0-30 cm) regardless of the species (Kleinhenz and Midmore 2001), we assume that roots of both bamboo types were within the same soil layer, so the characteristics of the soil in contact with roots were similar.

Differences in Si accumulation may be attributed to differences in the silicon uptake capacity of roots. Recently, two genes (*Low silicon rice 1*: *Lsi1* and *Lsi2*) encoding silicon transporters were identified in *japonica* rice (Ma and Yamaji 2008). Ma et al., (2007) have shown that genotypic differences between two rice species, *japonica* and *indica,* were due to the difference in abundance of Si transporters in rice roots. Therefore, differences between bamboos species, and

to a further extent between the two types of bamboo could reflect a difference in expression of genes responsible for silicon uptake.

After root uptake, Si is translocated to the shoot via the xylem. It is likely that transporters are also required for xylem Si loading and unloading and for distributing Si to the above-ground plant parts (Ma and Yamaji 2008). Genetic differences may also be expressed in the relative distribution of Si in stems and leaves. For example, Keeping et al., (2009) found that cultivar differences in sugarcane stalks could be explained by differing propensities of cultivars to deposit Si within the stalk epidermis. Active processes could partly explain differences in Si concentrations between species, but the understanding of the silica deposition process and the identification of transporters in plant shoots still need to be studied.

In the above-ground parts of bamboos, Kleinhenz and Midmore (2001) highlighted that the lifespan of leaves was substantially different between monopodial and sympodial bamboos. The canopy of monopodial species is rejuvenated every year when 2-year-old leaves are replaced by new ones. Those of sympodial species remain on culms longer, i.e. up to about 6 years. Therefore culms of sympodial bamboos of over 2-years-old contain relatively older and less productive leaves than monopodial bamboos. Motomura et al. (2002) reported that in bamboo leaves silica is continuously accumulated in the tissues throughout their life. Leaves of sympodial bamboos may therefore have a higher Si content than leaves of monopodial bamboos. However, in this study, we only sampled 1-year-old stems, so the leaf ages should all have been the same. The differences observed in our study between monopodial and sympodial bamboos were thus not related to this character.

4.3. Origin of Cu and Zn variability in bamboo

Due to the lack of published Zn and Cu concentrations in bamboos, we first compared our results with another Poaceae species, i.e. sugarcane (stems), growing in Réunion on similar soils (Collin and Doelsch 2010). Copper concentrations in bamboo samples were higher than in sugarcane samples, with an average of 3.5 ± 1.0 and 4.5 ± 2.0 mg kg^{-1} at the stem base and tip, respectively (Table II.2), whereas in sugarcane the average was 2.1 ± 0.6 mg kg^{-1}. Zinc concentrations in bamboo stems were similar to concentrations in sugarcane stems, with an average of 7.0 ± 6.3 and 14.8 ± 16 mg kg^{-1} at the bamboo stem base and the tip, respectively (Table II.2), and 10 ± 5.2 mg k^{-1} in sugarcane. We then compared leaf concentrations with data compiled for mature leaf tissue from various plant species (Kabata-Pendias and Mukherjee 2007). The concentration ranges that these authors considered normal were 5-30 mg kg^{-1} for Cu and 27-150 mg kg^{-1} for Zn. The concentration measured in bamboo leaves were within this range for Cu, with an

average of 5.1 ± 1.0 mg Cu kg^{-1}, and lower for Zn, with an average of 15.7 ± 5.6 mg Zn kg^{-1} (Table II.2). Cu and Zn concentrations in above-ground parts of Réunion bamboos were thus relatively low, which may confirm the low phytoavailability of these elements measured by NH_4NO_3-extraction, as well as the nonspecific ability of bamboos to accumulate Cu and Zn.

Within the stem, Cu and Zn concentrations, similar to Si, significantly increased from the stem base to the tip (Table II.2), suggesting that part of this element translocation was driven by transpiration. However, metals do not move freely in the plant. Interactions of cations with negatively charged sites (mainly with pectins) in xylem or phloem cell walls lead to decoupling of ion transport and water flow (Franco et al. 2002). In addition, most metals are complexed by organic acids, amino acids, peptides, metallothionine or phytochelatin (Broadley et al. 2007; Cobbett and Goldsbrough 2002; Liao et al. 2000). The greater Zn accumulation at the stem tip as compared to that of Cu may thus be explained by differences in the mobility of metal complexes formed within the plant.

Significant differences between species were measured for Cu in stems and leaves and for Zn in leaves (Table II.2). This finding is in good agreement with the study of Broadley et al. (2007) who reported that, in a dataset of 365 species, there were substantial differences in shoot Zn content between and within genera and species. Metal absorption in plants is both active and passive, and metabolic mechanisms, such as the expression of specific transporters in root cells, is genetically controlled and may thus vary between species. For example, the expression and gene organization of proton pumping ATPase genes in the plant plasma membrane seems to differ between species (Morsomme and Boutry 2000). Moreover, the availability of elements in the soil and root uptake can be affected by plant factors such as root exudates, root surface area, root absorption ability and mycorrhization (Keller et al. 2003; Langer et al. 2010; Whiting et al. 2000). For example in rice, Zn uptake efficiency is correlated with exudation rates of low molecular weight organic anions and a substantial proportion of the phenotypic variation in Zn uptake efficiency is under genetic control (Wissuwa et al. 2006). In wheat, genotypic variations in Zn uptake may be related to the release of phytosiderophores (mugineic and avenic acids), which were shown to significantly enhance Zn bioavailability (Cakmak et al. 1996; Rengel et al. 1998; Tolay et al. 2001). Thus, these mechanisms and inherent differences in uptake, translocation and accumulation may partly explain the significant differences noted in Cu and Zn concentrations among the 16 bamboo species.

Differences between monopodial and sympodial bamboos were significant for Cu and Zn, but no information is available in the literature on any possible specific behaviour regarding metals

between the two types of bamboo. Analyses of the differences with Si content were discussed above, and may also apply for Cu and Zn.

4.4. Interactions between metals and Si

No correlations between Cu, Zn and Si contents were found in stems and leaves. In the natural pedo-climatic environment of Réunion, with a soil having low NH_4NO_3-extractable Cu and Zn, the Si and trace metal behaviours in bamboos seem to be independent. This could be easily explained since the beneficial effects of Si are usually expressed when plants are subjected to stress conditions (Liang et al. 2007). It has been shown, for example, that Si alleviates Zn toxicity in heavy metal-tolerant *Cardaminopsis halleri* (Neumann and zur Nieden 2001). These authors suggested that the formation of Zn-silicate compounds in the cytoplasm may be responsible for the alleviation of the Zn toxicity. In *Arabidopsis thaliana*, Li et al. (2008) have shown that Si improves the resistance to Cu stress. A recent study has shown that Si modulates the expression of various genes involved in Cu tolerance in *A. thaliana* (Khandekar and Leisner 2011). Our study was not designed to assess the interaction between Si and metals at the cellular level, but we can assume that, with such a high Si absorption capacity in its tissues, bamboo may be able to tolerate higher metal concentrations than those present in Réunion soils. However, this would need further testing with increasing metal concentrations.

In conclusion, we report the first data on Cu and Zn concentrations in stems and leaves of various bamboo species and new data on Si in bamboos. Our results highlight the importance of the variability between species, particularly between monopodial and sympodial species for Zn, Cu, and Si contents in bamboos, suggesting that a genotypic character may be responsible for their accumulation.

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5. BILAN DU CHAPITRE II

Le **silicium est majoritairement accumulé dans les feuilles**, au sein desquelles sa concentration varie entre 82±2,5 et 159±2,3 mg.g^{-1} de MS. Les concentrations en **Cu et Zn** mesurées dans les bambous sont **relativement faibles**, elles sont comprises entre 2,0±0,3 et 7,6±1,8 mg Cu kg^{-1} de MS et entre 2,3±0,5 et 41±40 mg Zn kg^{-1} dans les chaumes et les feuilles.

Une partie de la variabilité observée est expliquée par des **différences d'accumulation** entre les 16 espèces de bambous. Nous avons montré que **les bambous sympodiaux**, présents dans les régions à climat tropical, **accumulent plus de Si et Cu** dans les chaumes et les feuilles, **que les bambous monopodiaux**. A l'inverse, les bambous sympodiaux accumulent moins de Zn dans les feuilles que les bambous monopodiaux.

Dans ces conditions pédoclimatiques naturelles, les comportements de Si et Cu dans le bambou semblent indépendants.

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CHAPITRE III

EFFECTS OF SILICON AND COPPER ON BAMBOO GROWN HYDROPONICALLY

TABLE DES MATIERES

CHAPITRE III

EFFECTS OF SILICON AND COPPER ON BAMBOO GROWN HYDROPONICALLY

L'un des objectifs principaux de ce travail de thèse est d'évaluer le rôle du silicium sur les bambous, en vue d'améliorer, si possible, leur croissance et donc leur productivité dans un système de phytoremédiation. Nous avons vu dans le chapitre précédent que les concentrations de Si accumulées dans les bambous sont très fortes, 109 $mg.g^{-1}$ en moyenne dans les feuilles. On peut donc se demander si la quantité de Si biodisponible dans le milieu va influencer leur croissance. Un déficit de Si affecte-t-il leur développement ? La croissance va t'elle être corrélée à la quantité de Si disponible ?

Sur un sol présentant des concentrations de cuivre phytodisponibles faibles, nous avons montré, dans le chapitre précédent, que les bambous accumulent des concentrations relativement basses de Cu dans leurs tissus : de 3,5 à 6,3 $mg.g^{-1}$ dans les feuilles. Dans ce contexte, les comportements de Si et de Cu dans le bambou semblent indépendants. Mais on peut se demander si les fortes concentrations de Si accumulées dans les bambous vont permettre d'améliorer la tolérance du bambou à de plus fortes concentrations de Cu. Jusqu'à présent les études montrant un effet de Si sur les métaux, ont été effectuées dans des conditions de forte toxicité métallique. Par exemple, Oliva et al. (2011) ont mis leurs plantes au contact d'une solution de 300 µM de Cu en culture hydroponique. Des concentrations de Cu libre si élevées ne sont pas mesurées en conditions environnementales, en raison de la forte affinité de Cu pour la matière organique présente dans le sol (Sauvé et al. 1997). Pour cette étude, nous avons choisi d'étudier le rôle de Si sur des bambous qui sont exposés à une concentration de Cu élevée mais cohérente avec des conditions environnementales.

Afin de contrôler les concentrations de Cu et Si apportées aux bambous, nous avons cultivé le bambou en hydroponie. Cette technique nous a permis de faire varier uniquement Si et Cu, de

suivre leurs concentrations dans les solutions nutritives et d'observer les effets de ces éléments sur les bambous.

Les objectifs de cette expérience sont les suivants :

- Localiser et quantifier Si dans les tissus du bambou exposés à une large gamme de concentration de Si dans la solution nutritive ;
- Evaluer l'effet de Si sur la croissance et le développement du bambou ;
- Evaluer l'effet de Si sur la concentration et la sensibilité au cuivre du bambou ;

L'article ci-après décrit l'expérience d'hydroponie qui a été réalisée pendant 11 mois. Nous avons choisi une espèce de bambou sympodial : *Gigantochloa* sp « Malay Dwarf » pour cette expérience. Les bambous ont été exposés à une large gamme de Si en solution (de 0 à 1,5 mM) pendant 6 mois, puis à une concentration potentiellement toxique de Cu (1.5 µM Cu^{2+}) pendant 2 mois.

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Effects of silicon and copper on bamboo grown hydroponically

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ABSTRACT

Due to its high growth rate and biomass production, bamboo is frequently used in industrial applications and has recently proven to be useful in wastewater treatment. Bamboo accumulates high silicon (Si) levels in its tissues, which may improve its development and tolerance to abiotic stresses, such as metal toxicity. This study investigates the beneficial effect of Si on bamboo growth and copper (Cu) sensitivity. A hydroponic culture of bamboo *Gigantocloa sp.* "Malay Dwarf " was performed for 8 months. The bamboo plants were first submitted to a range of Si supplementation (0 to 1.5 mM) that was added to a potentially toxic Cu concentration for plants (1.5 µM Cu^{2+}) after 6 months.

Silicon affected neither the development (number of stems and leaves, height and total dry mass) nor the nutrient content of the bamboo plants. The Si concentration in the plants increased markedly with an increasing Si concentration in the nutrient solution. The distribution

of Si among the different plant parts in all of the Si treatments was as follows: root < stem < leaf. In the leaves, the Si concentration ranged from below the detection limit to 218 ± 19 mg g^{-1}, which is one of the highest Si values of Si found that have been observed in bamboo plants that were grown in nature. After each renewal of the nutrient solution, the Si content in the solution decreased progressively, whereas the Cu concentration decreased abruptly during the first hours and then remained constant in the solution. These differences in Cu and Si uptake may indicate that bamboo takes up and allocates these two elements differently. The Cu addition did not induce any toxicity symptoms in the bamboo plants. The absorption of Cu in the bamboo plants was not altered by the Si supplementation; Cu accumulated mainly in roots (131 ± 39.3 mg kg^{-1}), which was followed by its accumulation in leaves (16.6 ± 3.6 mg kg^{-1}) and stems (9.8 ± 3.8 mg kg^{-1}).

Hydroponic culture has proven to be an efficient technique to grow the sympodial bamboo *Gigantocloa sp.* "Malay Dwarf". Bamboo growth does not depend on Si levels even though the bamboo *Gigantocloa sp.* "Malay Dwarf" did absorb Si in large amounts. The different absorption mechanisms for Cu and Si may partially explain why Si did not influence the Cu repartition and concentration in bamboo. Given the high biomass and its adsorption capacity, the bamboo tolerates high Cu concentrations in nutrient solutions.

Keywords: hydroponics, metal, silicon accumulator, Poaceae

1 INTRODUCTION

Extensive agronomic evidence supports the beneficial effects of silicon (Si) on the growth, development and yield of plants and the protection of plants against biotic and abiotic stresses (Cooke and Leishman 2011; Epstein 1994; Epstein 1999). Bamboo species that contain large deposits of silica in the leaves (Motomura et al. 2004) are classified among the silicon-accumulating plants (Ma and Takahashi 2002). Although Si distribution in bamboo has been described in several studies (Collin et al. 2011; Ding et al. 2008b; Li et al. 2006; Lux et al. 2003), the effects of Si on bamboo growth are poorly described (Ma and Takahashi 2002). Bamboo species are commonly found in temperate, tropical and subtropical regions, and they are widely used for industrial purposes, as fresh edible shoots, for making paper, as building material and even in medicine. They are known for their resistance to a wide range of stress factors, their high growth rate and their high biomass production, and they have potential uses in phytoremediation. The PHYTOREM company (France) has developed BAMBOO-ASSAINISSEMENT® technology for wastewater treatment (Arfi et al. 2009). Therefore, the potential beneficial effects of Si on bamboo may be used to optimise the phytoremediation capacity of bamboo.

For various reasons, there is increasing concern regarding copper (Cu) toxicity in agriculture. These concerns involve the high soil Cu contents that are caused by the long-term use of copper–containing fungicides (e.g., in vineyards) (Michaud et al. 2007), industrial and urban activities (air pollution, city waste and sewage sludge), and the application of pig and poultry slurries that are rich in copper (Legros et al. 2010; Marschner 1995).

Silicon is known to play a significant role in minimising the toxic effects of metals. Several mechanisms that are responsible for metal-stress alleviation have been proposed, such as the modification of the metal distribution inside the plant, an increase of metal binding to cell walls, and the co-precipitation of Si-metal (Rogalla and Romheld 2002). These studies have primarily focussed on Cd, Zn, Mn and Al, but Cu has been poorly studied to date. Two recent studies regarding the Cu-tolerant species *Erica andevalensis* and *Arabidopsis thaliana* have described the beneficial effects of Si on Cu toxicity (Khandekar and Leisner 2011; Li et al. 2008; Oliva et al. 2011). Oliva et al. (2011) reported that addition of Si reduced the leaf metal concentration, while Li et al. (2008) suggested that Si affects metal distribution or bioavailability. The Cu concentration in bamboo and its interaction with Si has been recently assessed (Collin et al.

2011). Collin et al. (2011) provided the first data regarding Cu concentrations in various bamboo species that were grown under similar natural pedo-climatic conditions; they showed that the Cu concentrations were relatively low, whereas Si was accumulated in the leaves at a level of 82 to 159 mg g^{-1} dry weight depending on the species. With such a high variability, it will be interesting to assess the extent of Si accumulation under increasing available Si concentrations and the effects that this supplementation may have on Cu uptake and toxicity. We chose to grow bamboo using hydroponics. The main advantage of this procedure is that plants can be grown under optimal nutrient conditions, and the nutrient supply can be adapted over time as necessary. To our knowledge, this is the first example of the application of this technique to bamboo. The questions that are addressed in this study are as follows. 1) How are bamboo growth and development affected by various Si supplementations? 2) How does the Si quantity and localisation in different bamboo organs change with Si supplementation? 3) Does Si supplementation modify the copper sensitivity of bamboo?

2 MATERIAL AND METHODS

2.1 Plant material, experimental design and pre-culture

Fifty one-year-old sympodial bamboo plants *Gigantocloa sp.* "Malay Dwarf" that were grown on the same substrate were provided by a bamboo nursery (A. PERRUSSOT, Le Guillaume, Saint Paul, Réunion Island). In most of the hydroponic experiments that have been performed with other species, seeds were directly used; however bamboo seeds are not typically used to propagate bamboo plants. Indeed, bamboo seeds are difficult to obtain and have a low germination rate. Most of the time, the propagation of bamboo is performed by vegetative reproduction, which involves culm cutting for sympodial bamboo and rhizome cutting for monopodial species. However, the growth from cutting is slow and produces non-homogeneous plants; therefore, we decided to use soil-grown bamboo and made *a posteriori* selections for their similar size and development so that we could work with a homogeneous population. The adaptation of the bamboo plants to hydroponic conditions was delicate because the bamboo roots were first adapted to soil. After a careful washing of the roots including removal of all of the soil particles, the bamboo plants were transferred into aerated nutrient solution tanks.

The experiment was performed for 11 months. A three-month pre-culture phase was conducted; during this period, the bamboo plants lost their leaves and began to develop new ones. The bamboo growth was conducted for 6 months (phase I). During this period, the bamboo plants

were supplemented with a large range of Si concentrations to observe the Si effect on bamboo growth. After the bamboo was well-developed with several news stems and leaves, Cu was added to the Si treatments for 2 months (phase II). This second phase was performed to observe the effect of Si on Cu concentrations in bamboo.

The composition of the pre-culture nutrient solution consisted of a 25 %-strength complete Hoagland solution. The composition of the macroelements (mM) was as follows: 1 $Ca(NO_3)_2$, 1.5 KNO_3, 0.24 $MgSO_4$, and 0.22 $(NH_4)_2HPO_4$; the composition of the microelements (µM) was as follows: 11.6 H_3BO_3, 0.08 $CuSO_4$, 0.2 $ZnSO_4$, 0.03 MoO_3, 2.3 $MnCl_2$, and 100 FeNaEDTA. The pH of this solution was adjusted to a pH of 6 with NaOH or HNO_3. The nutrient solution was renewed every 7 days. Analytical-grade chemical reagents and ultrapure water were used to prepare the nutrient solutions. No glassware was used, which minimised Si contamination. All of the plasticware that was used in the experiments was previously soaked overnight in nitric acid and rinsed with ultrapure water. The growth chamber parameters were set to the following (day/night): 28/24°C, 90/70 % relative humidity and 450 µmol photons m^{-2} s^{-1} light intensity.

2.2 Experiment in nutrient solution

2.2.1 Phase I: Si treatment

At the end of the pre-culture stage, 25 bamboo plants of similar sizes were selected and transferred to individual pots. The plants were then grown in cylindrical PVC pots that contained 2.5 L of nutrient solution. For 6 months, from day 0 to day 191, five Si treatments were applied as follows: 0.0 (control), 0.38, 0.75, 1.13 and 1.5 mM, which are referred to as Si0, Si04, Si08, Si1.1, and Si1.5, respectively. These concentrations were chosen to cover a large range of environmentally relevant Si concentrations. Indeed, in natural soil solutions, orthosilicic acid (H_4SiO_4) concentrations range from 0.1 to 1.7 mM (Knight et al. 2001). Si was provided as a monosilicic acid from potassium metasilicate $Si(KOH)_2$, which has a molar K:Si ratio of 2 (Metso 400 - YARA) (Voogt et al. 2001). The potassium and hydroxide levels were adjusted to compensate for the additional input of K and OH from the addition of Si. The composition of the macroelements (mM) in this modified nutrient solution was therefore 0.35 $Ca(NO_3)_2$, 0.3 $CaCl_2$, 0.24 $MgSO_4$, and 0.22 $(NH_4)_2HPO_4$, and the composition of microelements (µM) was 11.6 H_3BO_3, 0.08 $CuSO_4$, 0.2 $ZnSO_4$, 0.03 MoO_3, and 6.5 $MnCl_2$. Fe was provided as 15 µM Fe-N, N9-di (2-hydroxybenzyl) ethylenediamine-N, N9-diacetic acid monohydrochloride hydrate (HBED; Strem chemical, USA). Fe(III)-HBED was prepared according to Chaney et al. (1998) in a manner such that all of the HBED was saturated with Fe. The KNO_3 and HNO_3

concentrations were adjusted in each treatment such that the concentrations of K and NO_3 reached 3 mM. The solution was set to a pH of 6.0 (±0.2) and was buffered with 1 mM 2-morpholinoethanesulphonic acid (MES).

Five replicates per treatment were conducted. The solution was continuously supplied by a peristaltic pump from the 15-L "reserve tank" to the base of the five 2.5-L pots, and the solution that exceeded 2.5 L was recovered via an overflow pipe and returned to the reserve tank. The nutrient solutions were continuously aerated with an air pump in each pot. All of the solutions from each pot and the reserve tank were completely renewed every 7 days.

The nutrient solution was sampled throughout the experiment at the beginning of the week (before contact with bamboos), referred to as "input solution". The solution was also sampled at the end of the week (just before the renewal), when it was referred to as the "output solution". The pH was measured in all of the solutions with a combined glass electrode. The solutions were then filtered (0.2 µm, Sartorius) and acidified to pH<2 using concentrated HNO_3, and they were refrigerated (4°C) before the analysis. The solutions were analysed for the presence of cations (Si, Ca, Fe, Mg, Mn, P, and Zn) by inductively coupled plasma-atomic emission spectrometer (ICP-AES Jobin Yvon J38).

2.2.2 Phase II: Cu + Si treatment

From day 191 until day 226 (2 months), 1.5 µM Cu was added to each Si treatment using $CuSO_4$. The Si and nutrient concentrations were maintained at levels that were identical to phase I. The Cu concentration was chosen because it had been previously shown to induce toxicity in *Poaceae* plants (Bravin et al. 2010), and it was consistent with Cu concentrations that were measured in contaminated soil (Sauvé et al. 1997). Due to the high bamboo biomass at this stage, which consequently required higher water uptake, the nutrient solutions were renewed every 5 days. Geochemical calculations were performed using PHREEQC (version 2-17) (Parkhurst 1995) and the MINTEQ database of thermodynamic constants to determine the Cu speciation in the input solution. The calculations indicated that free copper should account for 97.6 % of the total Cu in the solution at pH 6. The quality of the speciation modelling was confirmed by measuring Cu^{2+} with a cupric ion selective electrode (Cu-ISE) (Orion 9629BNWP). The ISE measurements were calibrated between pCu^{2+} 9.0 and 5.2 in a 10^{-4}M Cu solution that was buffered with iminodiacetic acid and potassium phthalate according to the procedure previously described by Rachou et al. (2007).

In phase II, 40-mL nutrient solution samples were taken from each of the input and output nutrient solutions. In addition, to more precisely evaluate the changes in the nutrient

concentrations during the 5 days (between the input and output solutions), the nutrient solution was also sampled from day 196 to day 201 at 1, 2, 3, 4, 5, 20, 23, 27, 43, 72 and 96 h after the solution was renewed. Additional samples were taken at day 216 1 h, 3 h and 44 h after the renewal of the solution.

All of the solutions were filtered (0.2 µm, Sartorius) and kept at a 4°C temperature. A subsample was acidified, and the solutions were analysed for the presence of cations (Si, Ca, Fe, Mg, Mn, P and Zn) by ICP-AES; the total Cu concentration was measured by inductively coupled plasma mass spectrometer (ICP-MS, Agilent 7500 CE). Free Cu^{2+} was analysed by Cu-ISE in another subsample immediately following the solution sampling. The Cu-ISE measurement was conducted on the following selected samples: in the input solution on day 196 and in the solutions that were sampled after 1, 2, 4, 27, 43, and 96 h; in the input solution on day 216 and at 1 h and 3 h; and in the output solution that was collected on day 221.

The Si and Cu uptake levels from the nutrient solution were calculated throughout the experiment. The uptake of Si or Cu corresponds to the quantity of Si or Cu that was measured in the input solution (mmol) minus the quantity of Si or Cu that was measured in the output solution (mmol). The elemental quantity (Si or Cu) in the solution is the element concentration that was measured by ICP-AES or ICP-MS multiplied by the volume of the solution. The volume of the input solution was 15 L, and the volume of the output solutions was measured throughout the experiment and varied between 13 and 8 L. In the figures that represent Cu and Si uptake (Figure III.2 and Figure III.3), the errors are estimated to be 20 %, and these errors were calculated based on the errors in the output volume of the solutions and the analytical errors. In the other figures (Figure III.1 and Figure III.4), error bars represent the uncertainty that was estimated from the analytical errors (ICP AES or ICP-MS).

2.3 Plant analysis

2.3.1 Growth parameters

The number and height of the stems and the number of leaves were assayed at the beginning of the experiment (day 0), at the end of phase I (day 191) and at the end of the phase II (day 226).

2.3.2 Analysis of tissues

At the end of phase II, all 25 plants were harvested. The plant leaves, stems, rhizomes and roots were separated. The samples were carefully washed with ultrapure water, and the fresh masses were determined.

Before the analysis, all of the parts were dried at 60°C until they reached a constant weight. The samples were subsequently mixed, ground and homogenised. The sub-samples were dried at 80 % until they reached a constant weight to determine their dry weight.

The plant samples (leaves, stems and roots) underwent dry mineralisation for the analysis of trace elements. During the mineralisation, the Si content was determined by gravimetry as follows: 500 mg of dried plant material was placed in a platinum dish and gradually heated to 500°C. The silica was eliminated in the ash with HF. After cooling, the Si weight was determined based on the difference from the 500°C weight. The ashes were dissolved into HCl, and the content of trace elements in the solutions was analysed by inductively coupled plasma-optical spectrometer (ICP-OES Vista-Pro, Varian, CIRAD, Montpellier, France). For quality control, in-house reference samples and certified samples (Astrasol-Mix, Analytika) were used every 20 samples, and each analysis was conducted in duplicate. The measurement uncertainty was less than 15 %. The quantification limit for Si was 5 mg g^{-1} of dry weight (DW).

The concentrations of macro- and micronutrients are expressed as g kg^{-1} or mg kg^{-1} DW, and the Si concentrations are expressed in mg g^{-1} DW SiO_2 such that the results are directly comparable with the literature.

2.4 Statistical analyses

The Minitab 15.1 software package (Minitab, Inc.) was used for the statistical analyses. For each portion of the plants, the concentrations of micro- and macronutrients were analysed by ANOVA. We used a one-way ANOVA at the 95 % confidence level with the Si treatments (5 levels) as the main factor, which was followed by a Tukey's post hoc test at the 95 % confidence level to evaluate the differences in the treatments. The mean concentrations of Si and Cu in the roots, stems and leaves were compared using a paired t-Test at the 95 % confidence level.

3 RESULTS

3.1 Evolution of the element concentrations in the nutrient solution

The concentrations of the nutrients that were measured in the output solutions differed from their concentrations in the input solutions. Table III.1 provides the Ca, Mg, P and Fe concentrations that were measured in the input and output solutions at three different time periods during the experiment. These time periods are used as an example because the variations throughout the experiment were reproducible. The Ca, Mg and Fe concentrations increased in the nutrient solution. The P concentration varied throughout the experiment; it increased slightly in the solution at the beginning of phase I from 0.22 mM to 0.24 mM in the input and output solutions, respectively (Table III.1), but after the day 135 during phase I, its concentration decreased in the output solution. Throughout the experiment, the Zn concentration remained constant (data not shown). The results of the extreme treatments Si0 and Si15 (Table III.1) illustrate that no Si treatment effect was observed on the uptake of the measured nutrients in either phase I or II. The variations of the macro- and micronutrients (Mg, Ca, P, Fe and Zn) throughout the two renewals (day 196 to 201) during phase II are shown in Figure III.1 . The increase in the Mg, Ca and Fe concentrations and the decrease of P and Zn were constant over time.

Despite the use of a pH buffer (MES), the pH of the solution changed over time (Table III.1). During the first 90 days of the experiment, the pH decreased and reached values between 5.5 and 5.9 in the output solution. From day 91 to day 191, the pH of the solution increased weekly and ranged from 6.3 to 7.3. During phase II, the pH consistently decreased following contact with the plants, and the final pH varied between 5.4 and 6.

Table III.1 **Ca, Mg, P, Fe and Zn concentrations in the nutrient solution with Si0 and Si1.5 treatments in the input and output solutions at three different time periods during the experiment**

	period (days)	treatment	pH		Ca (mM)		Mg (mM)		P (mM)		Fe (µM)	
			input solution	output solution	input solution	output solution	input solution	output solution	input solution	output solution	input solution	output solution
Phase I *Day 0 to 191*	29-36	Si0	6	5.7	0.67	1.24	0.23	0.28	0.22	0.24	16.08	17.49
		Si1.5	6	5.6	0.65	1.15	0.24	0.29	0.22	0.24	17.13	18.37
	126-133	Si0	5.8	6.5	0.61	1.03	0.21	0.31	0.19	0.19	15.14	21.18
		Si1.5	5.8	6.3	0.61	0.83	0.21	0.27	0.18	0.17	14.34	19.88
Phase II *Day 191 to 226*	216-221	Si0Cu	5.9	5.5	0.62	0.74	0.22	0.24	0.22	0.17	15.72	18.72
		Si1.5Cu	5.8	5.4	0.6	0.76	0.23	0.26	0.21	0.18	14.53	17.76

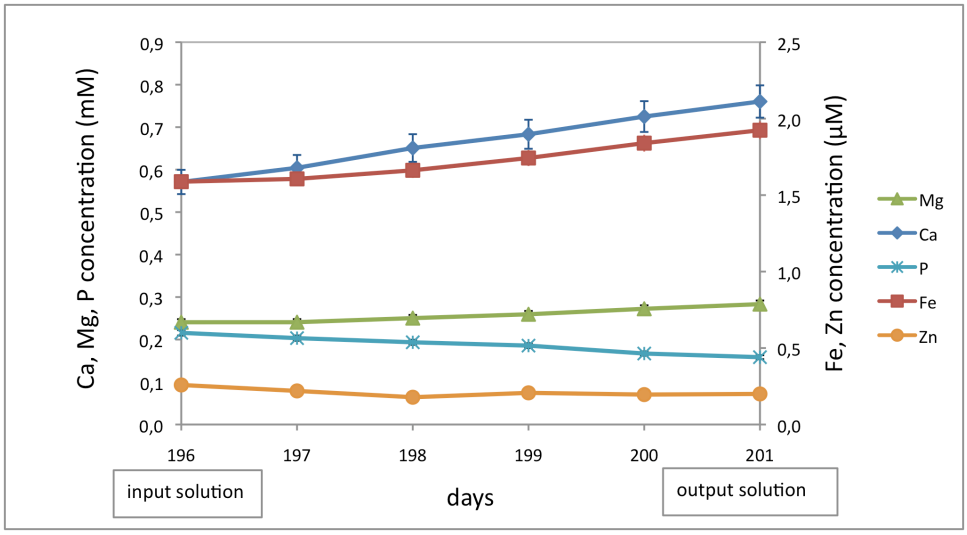

Figure III.1 **Concentrations of macronutrients (Ca, Mg and P) and micronutrients (Fe and Zn) in the Si0.4 treatment during phase II from days 196 to 201.**

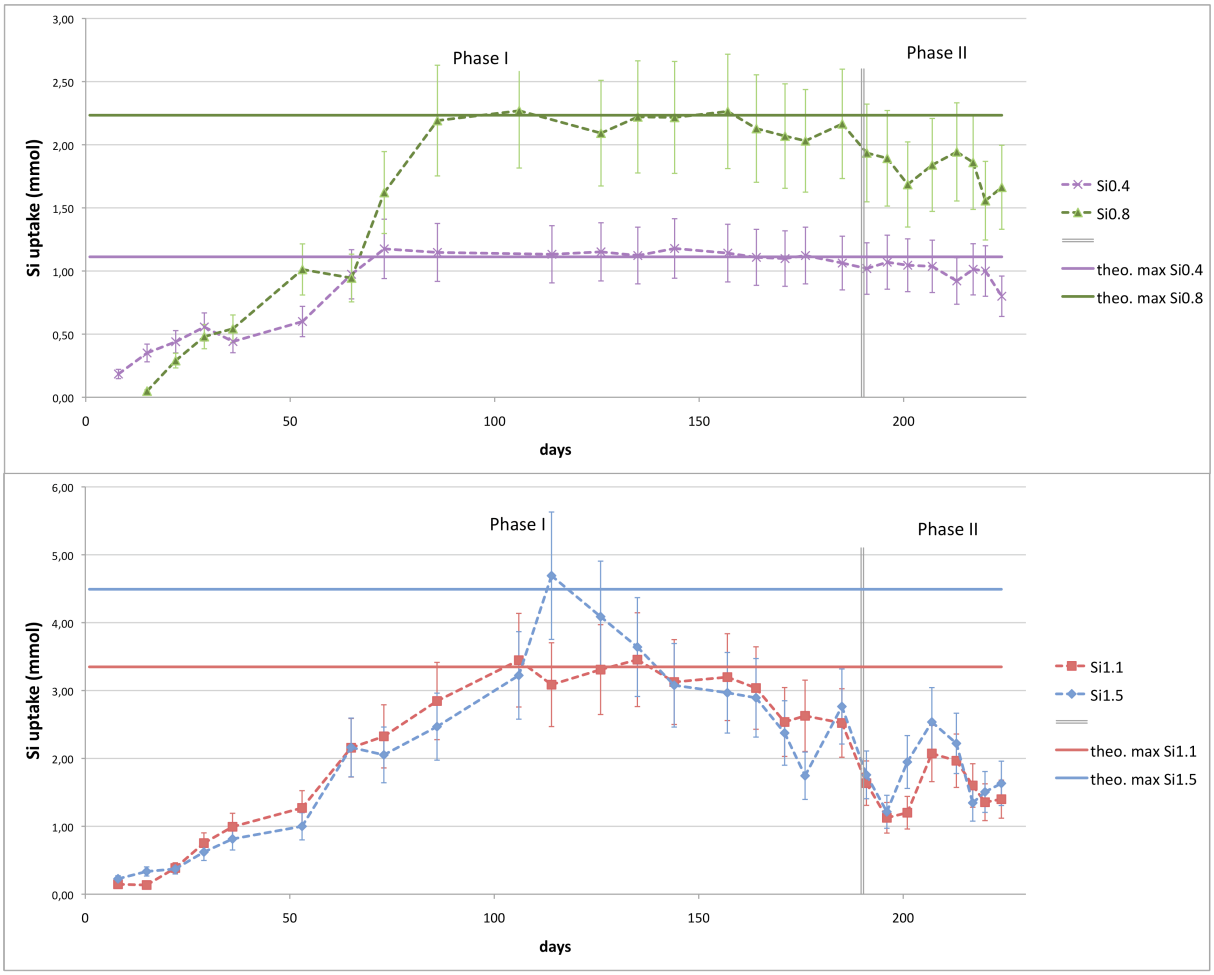

Figure III.2 **Bamboo Si uptake throughout the experiment, which was calculated as the difference between the Si concentrations that were measured in the input and output solutions. "Theo. max" is the theoretical maximum uptake that could be obtained if the entire Si in solution is taken up by the plants.**

The silicon concentrations in the solution decreased during the contact with bamboo. Si uptake from the Si concentrations in the input and output solutions was calculated for each renewal, and the evolution throughout the experiment is shown in Figure III.2 . For all of the treatments, at the beginning of the experiment, the uptake increased until it reached a plateau. This plateau indicates that the solution was entirely depleted during its contact with the bamboo. The Si content in the solution was initially depleted in the less-concentrated solutions, starting from day 65, 73 and 106 for the Si0.4, Si0.8 and Si1.1 treatments, respectively. The uptake then decreased starting on days 176, 157 and 164 for the Si0.4, Si0.8 and Si1.1 treatments, respectively. The Si uptake in the higher treatment (1.5 mM) increased until day 114, and it then decreased until day 176. The Si uptake was similar in the Si1.1 and Si1.5 treatments, with the exception of the period between days 114 and 126. This uptake decrease was observed, although nothing was changed in the experiment. Cu was added to the solution beginning on day

191. During phase II, the Cu that was added to the solution did not modify the downward trend of Si uptake in the Si0.8, Si1.1 and Si1.5 treatments. However, in these treatments, the Si uptake was variable throughout phase II. In the Si0.4 and Si0.8 treatments, Cu seemed to slightly decrease the Si uptake.

The Cu uptake differences between the Si treatments during phase II were not significant (Figure III.3), and the Cu concentration in the output solution throughout phase II varied between 0.72 and 0.91 µM. During the contact with the bamboo plants, i.e., for 5 days, the Si and Cu concentrations both decreased (Figure III.4), unlike the Ca, Mg and Fe concentrations (Figure III.1). The rate of decrease was different for Si and Cu; the Si concentration gradually declined in the solution over the period of 5 days, whereas the Cu concentration declined sharply in the first hours after the nutrient solution was renewed (Figure III.4). The total Cu concentration that was measured in solution by ICP-MS correlated well with the pCu value that was measured by ISE (R^2=0.7) (data not shown).

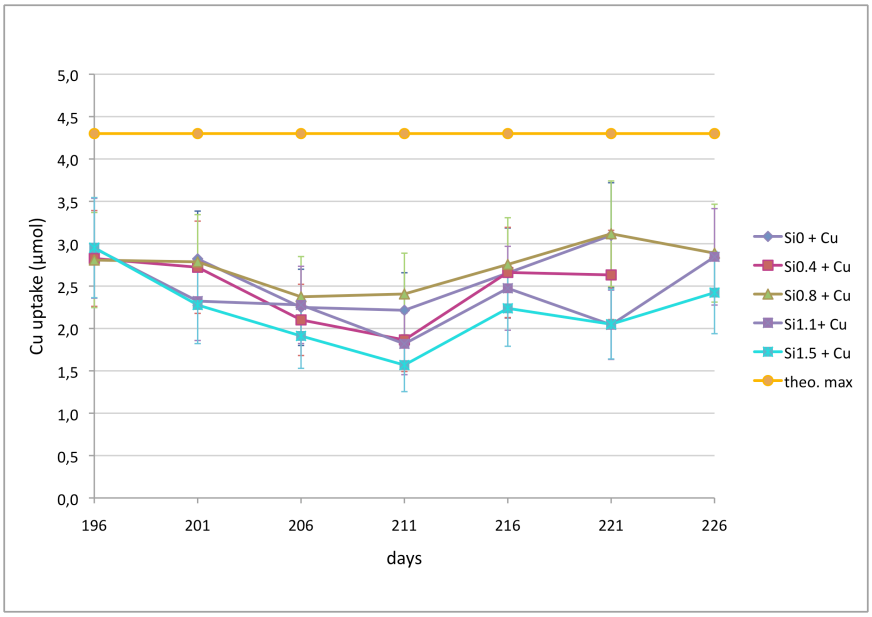

Figure III.3 **Bamboo Cu uptake throughout the experiment, which was calculated from the difference in the Cu concentrations in the input and output solutions. "Theo. max" is the theoretical maximum uptake that would be obtained if all of the Cu in the solution was taken up by the plants.**

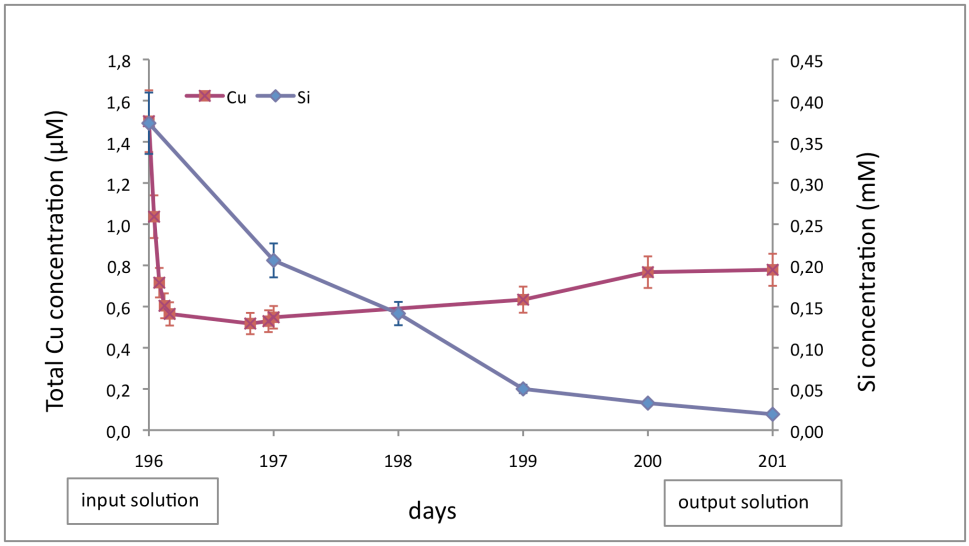

Figure III.4 **Si and Cu concentrations in the nutrient solution during the Si0.4 treatment in phase II from days 196 to 201.**

3.2 Bamboo growth parameters and elemental distribution

At the end of phase I (day 191), the number of stems and leaves was similar between the different Si treatments (Table III.3). The number of leaves varied between 170 ± 117 in the Si1.5 treatment and 377 ± 243 in the Si0.8 treatment. At the end of phase II (day 224), no significant differences in the plant growth parameters, including the final fresh weight content (g), the number of stems and their heights (cm), and the number of leaves, were observed between the Si treatments (Table III.3). The numbers of leaves and stems that developed during phase II were also similar between the treatments. At the harvest, each bamboo plant contained an average of 20 ± 14 stems that were 20 ± 4.5 cm high and had a total of 241 ± 168 leaves (Table III.3). The fresh matter from the final harvest was not significantly affected by the Si treatments.

The Si concentrations in the bamboo plants are presented in Figure III.5 . The Si content in the leaves varied from below the detection limit (5 mg g^{-1}) for the Si0 treatment to 218 ± 19 mg g^{-1} for the Si1.5 treatment. The Si content in the stems varied from below the detection limit in the Si0 treatment to 65 ± 14 mg g^{-1} for the Si1.5 treatment, and the root Si content ranged from below the detection limit for the Si0 treatment to 11 ± 3 for the Si1.5 treatment. Our results demonstrate that the Si concentration in bamboo *Gigantocloa sp.* "Malay Dwarf" decreased in the following order: leaf > stem > root. The larger Si content that was measured in the nutrient solution resulted in highly significant linear increases in the Si concentrations from 0.4 mM to 1.5 mM (R^2>0.99 for the leaves). The slope of the regression line between the Si content in the solution and the Si concentration in the bamboo parts was different between 0 and 0.4 mM and

between 0.4 mM to 1.5 mM Si in solution. Due to the uncertainty in the bamboo Si concentration in the Si0 treatment (which was below the detection limit), we have drawn a dotted line between Si0 and Si0.4. The nitrogen, P, K, Ca and Mg concentrations were affected by the Si treatment (Table III.2). These concentrations declined significantly in response to increasing Si concentrations. The Fe, Mn and Zn concentrations were not affected by the Si treatments.

At the end of phase II, no Cu toxicity symptoms (chlorosis, major root damage or growth inhibition) were observed regardless of the Si treatment. The Si treatments did not cause any significant effect on the Cu concentrations in the plants. Because Si did not influence the Cu concentration in any of the plant parts, the Cu concentrations in the different treatments were averaged in Figure III.6 . Copper accumulated in the plants in the following order: root > leaf > stem; the averages were 131 ± 39.3 mg kg^{-1} in the roots, 16.6 ± 3.6 mg kg^{-1} in the leaves and 9.8 ± 3.8 mg kg^{-1} in the stems.

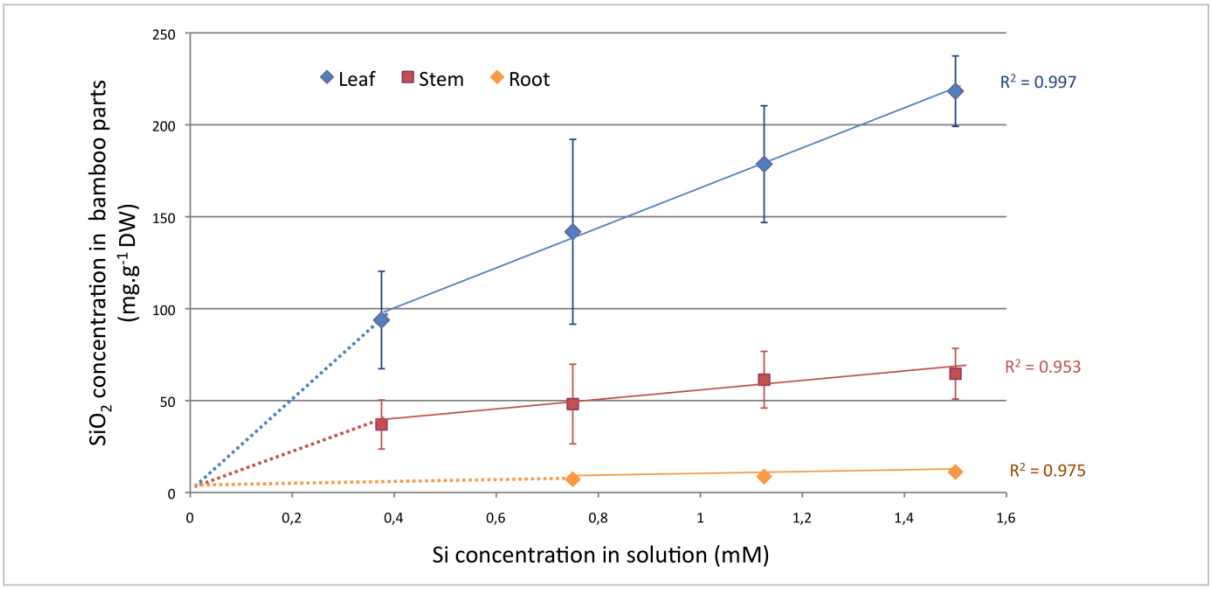

Figure III.5 **Si concentrations in different bamboo organs as a function of the Si concentration in the nutrient solution at 1.5 µM Cu. The error bars represent the standard deviation of the SiO_2 concentrations in the 5 different bamboo plants.**

Table III.2 **Concentration of macro and micronutrients in the leaves, stems, and roots of the bamboo plants that were grown with different Si concentration in the nutrient solution**

	treatment	Dry matter (%)		N		P		K		Ca		Mg		Fe		Mn		Zn	
				mean (mg.g^{-1} DW)										mean (mg.kg^{-1} DW)					
Leaves	Si0	34.8 ± 6.0	a	31.28 ± 2.1	a	8.0 ± 1.1	a	19.9 ± 2.3	a	10.9 ± 1.6	a	3.9 ± 0.4	a	75 ± 30	a	882 ± 288	a	50 ± 29	a
	Si0.4	35.5 ± 3.5	a	27.7 ± 1.9	ab	6.4 ± 1.8	ab	17.8 ± 2.9	ab	10.4 ± 0.5	ab	3.4 ± 0.1	b	27 ± 5.9	a	561 ± 160	ab	45 ± 16	a
	Si0.8	39.7 ± 2.3	a	27.7 ± 2.8	ab	5.1 ± 2	b	15.3 ± 1.7	b	9.4 ± 2.2	abc	2.9 ± 0.35	c	34.5 ± 15.2	a	421 ± 234	b	33 ± 18	a
	Si1.1	37.5 ± 7.4	a	25.9 ± 1.2	b	6.3 ± 1.0	ab	17.9 ± 2.8	ab	8.4 ± 0.7	bc	2.6 ± 0.1	c	26.6 ± 5.5	a	413 ± 175	b	43 ± 9.3	a
	Si1.5	38.7 ± 2.7	a	25.7 ± 1.9	b	5.7 ± 0.9	ab	16.1 ± 2.1	ab	7.4 ± 0.9	c	2.5 ± 0.1	c	25.3 ± 5.3	a	472 ± 103	b	49 ± 16	a
Roots	Si0	15 ± 5.2	a	28.4 ± 3.3	a	4.2 ± 0.9	a	17.1 ± 3.8	a	0.73 ± 0.2	a	1 ± 0.1	a	389 ± 82.5	a	647 ± 283	a	37 ± 5.9	a
	Si0.4	12.7 ± 1.7	a	25.1 ± 1.3	a	3.3 ± 0.3	a	13.2 ± 1.7	a	0.73 ± 0.2	a	0.8 ± 0.1	a	347 ± 70	a	886 ± 609	a	37 ± 8.3	a
	Si0.8	13.7 ± 2.8	a	26.8 ± 2.4	a	3.2 ± 0.8	a	14.5 ± 4.1	a	0.72 ± 0.3	a	0.9 ± 0.2	a	357 ± 96	a	504 ± 335	a	40 ± 7.5	a
	Si1.1	12.3 ± 0.9	a	25.4 ± 2.1	a	4.1 ± 0.7	a	16.2 ± 2.5	a	0.5 ± 0.1	a	0.8 ± 0.1	a	424 ± 83	a	629 ± 153	a	34 ± 3.0	a
	Si1.5	16.4 ± 3.2	a	25.9 ± 1.2	a	4.2 ± 0.7	a	15.9 ± 3.9	a	0.5 ± 0.1	a	0.9 ± 0.1	a	417 ± 87	a	846 ± 385	a	33 ± 3.6	a

*DW : dry weight ; ** For each part, different letters indicate significant means differences by Tukey test

Table III.3 **Effect of different levels of Si nutrition on the following growth parameters: number of stems and leaves that developed during the experiment in phase I and phase II.**

Treatment	Leaves			Stems			Height (cm)		Fresh matter (g)
	Day 0	192th days	224th days	Day 0	192th days	224th days	Day 0	224th days	224th days
Si0	15 ± 3.2 a	132 ± 100 a	172 ± 145 a	4.4 ± 3.4 a	10 ± 11 a	15 ± 15 a	19 ± 7.7 a	17.8 ± 3.7 a	115 ± 112 a
Si0.4	18 ± 9.5 a	238 ± 128 a	258 ± 201 a	5 ± 1.9 a	14 ± 3.3 a	17 ± 7 a	24 ± 7.7 a	22.8 ± 4.0 a	148 ± 111 a
Si0.8	22 ± 15 a	351 ± 204 a	376 ± 243 a	4.6 ± 1.7 a	23 ± 15 a	34 ± 21 a	21 ± 7.1 a	16.9 ± 5.9 a	177 ± 127 a
Si1.1	19 ± 12 a	175 ± 62 a	226 ± 75 a	4.6 ± 2.4 a	16 ± 7.1 a	20 ± 10 a	23 ± 5.9 a	20.9 ± 3.6 a	145 ± 82 a
Si1.5	17 ± 9.4 a	165 ± 134 a	170 ± 117 a	4.6 ± 1.3 a	14 ± 12 a	15 ± 12 a	19 ± 3.9 a	21.6 ± 2.9 a	99 ± 95 a
mean	18 ± 8.1	212 ± 143	241 ± 168	4.6 ± 2.0	15 ± 10	20 ± 14	21 ± 6.3	20 ± 4.5	137 ± 99

. *For each part, different letters indicate significant means differences by Tukey test

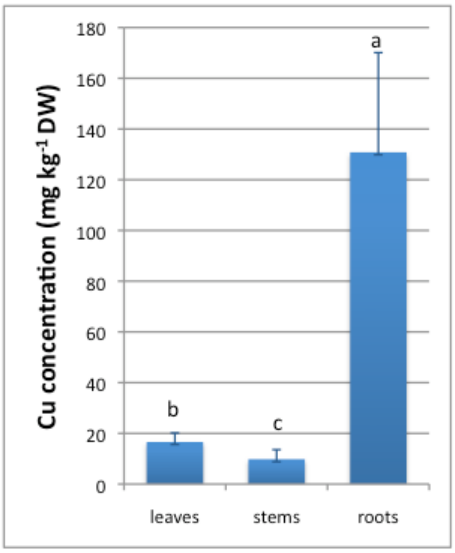

Figure III.6 Repartitioning of the Cu concentration in bamboo plants.

4 DISCUSSION

4.1 Silicon in bamboo

4.1.1 Mechanisms of silicon accumulation

The Si uptake varied greatly throughout the experiment. The increased uptake during the first 106 days that is shown in Figure III.2 is likely associated with biomass production. At the beginning of the experiment (day 0), the bamboo plants contained an average of 18 leaves (Table III.3), and they developed until they each contained between 124 and 324 leaves at harvest. Si uptake decreased beginning on day 114 in the Si1.5 treatment and day 164 in the Si0.4, Si0.8 and Si1.1 treatments. One hypothesis that would explain this decrease is that the plants developed at a slower rate. Indeed, the development of the bamboo plants and, particularly, the roots may have been inhibited by the pot size.

During phase I, the pH of the output solution ranged from 5.5 to 7.3 (Table III.1). The pH decreased at the beginning of the experiment during plant contact, whereas after 90 days, the pH increased. The pH in the solution seems to correlate with the bamboo development. The pH is an important property in the regulation of solubility and the speciation of elements in the nutrient solution. However, at pH 8, Si speciation is not affected, and Si is primarily present as an orthosilicic acid in the nutrient solution, which should not modify Si uptake.

Silica concentrations in the leaves and stems of bamboo *Gigantocloa sp.* "Malay Dwarf" that is grown in hydroponics can be compared with data that have been obtained from field-grown plants (Collin et al. 2011). The same species that were grown under natural conditions contained a leaf Si content of 135 mg g^{-1} and a stem Si content that was between 5.9 mg g^{-1} (base) and 30 mg g^{-1} (tip of the stem). These concentrations are similar to those that were measured in the Si0.4 and Si0.8 treatments; the leaves contained 94 and 142 mg g^{-1} Si, respectively, and the stems contained 37 and 49 mg g^{-1}, respectively. The Si concentrations in the leaves and stems of the Si1.1 and Si1.5 treatments were higher than those that were measured in all of the studied bamboo species as reported by Collin et al. (2011), who reported a Si range of 55 to 159 mg g^{-1} SiO_2 in the leaves of 16 temperate and tropical bamboo species. The Si concentrations in the leaves reached an average of 218 mg g^{-1} in the highest Si treatment, which was higher than any Si content that has been previously reported in the literature (Ding et

al. 2008b; Li et al. 2006; Lux et al. 2003) with the exception of Motomura et al. (2002). The authors reported a Si content of 410 mg g^{-1} in the 3-year-old leaves of the bamboo *Sasa veitchii*, while in our study, the Si content was measured in bulk from a maximum of 8 month-old leaves. In all of the treatments, the root silica content was always lower than the stem and leaf silica contents (Figure III.5). This pattern is different from the pattern that was found in previous studies concerning Si in bamboo, which showed that the root Si content was higher than that of the stems but lower than those of the branches and leaves (Ding et al. 2008b; Lux et al. 2003). These previous studies were performed on bamboo that was grown in soil, and the root-cleaning procedure may have been inefficient at removing all of the Si-containing soil particles. However, this possibility can be ruled out for the study of Ding et al. (2008b), who attempted to reduce the potential bias throughout the cleaning procedure. However, this concentration pattern is consistent with previous studies that were performed in hydroponics with two other *Poaceae*, rice (Ding et al. 2008a) and banana (Henriet et al. 2006; Opfergelt et al. 2006). In these species, the concentrations also increased from the roots to the stems and the leaves.

After Si was supplied in solution, a significant linear relationship between the tissue (leaf, stem and root) Si content and the nutritive Si concentration was observed (Figure III.5). This Si distribution in the different parts of the plant suggests passive uptake. Si translocation within the plant is largely dependent on the transpiration stream, which carries silicic acid and deposits it in the form of amorphous silica (Jones et al. 1967); this primarily occurs in the leaves. In the leaves and roots, the Si content did not reach a plateau, which indicates that the Si uptake can increase even more; this is contrary to rice, which reached maximum Si uptake at a Si concentration greater than 1.28 mM (Ma and Takahashi 2002). However, as Si may polymerise in solution at concentrations above 2 mM, it is not possible to test larger Si concentrations and thus possibly observe a larger uptake by bamboo. The only possibility would be to increase the length of the experiment under the hypothesis that bamboo can take up Si more quickly than it can grow.

Recent studies have demonstrated the existence of active Si transporters in roots (Lsi1 and Lsi2), which are responsible for the high Si uptake capacities of rice and maize (Mitani et al. 2009; Yamaji and Ma 2011). Therefore, Si uptake in Si accumulators is explained by the coexistence of active and passive uptake mechanisms (Ding et al. 2008b; Ma et al. 2007; Mitani et al. 2009). In our study, the depletion of Si in the nutrient solution of the Si0.4 and Si0.8 treatments indicates that silicon, which is transported together with water, is not the only absorption mechanism; this further suggests that an active process is involved in Si uptake (Henriet et al. 2006; Rafi and Epstein 1999). Therefore, the coexistence of passive and active

uptake and transportation mechanisms would be consistent with previous studies on bamboo, rice and wheat (Ding et al. 2008b; Ma and Yamaji 2008; Motomura et al. 2004).

4.1.2 Effect of Si uptake on the nutrient concentration and growth parameters

The variations in the macro- and micronutrient (Mg, Ca, P, Fe and Zn) concentrations throughout the two renewals (Figure III.1) were attributed to both plant uptake and the decrease in the solution volume due to evaporation and water uptake (water decrease of 13 % at the beginning and 47 % at the end of the experiment). The variation of the P concentration in the output solution throughout the experiment (i.e., an increase that was followed by a decrease from day 135 in the output solution) indicates that the nutritional need of the bamboo plants increased after day 135. The Si supply did not affect the uptake of any of the measured nutrients (Table III.1). However, the addition of Si significantly reduced the leaf concentrations of macronutrients (N, P, K, Ca and Mg) (Table III.2). The decrease in these concentrations is correlated to the increase in Si in the leaves. In fact, the high amount of silica that precipitated in the leaves can explain the decrease in the nutrient concentrations. Indeed, if we subtract the quantity of SiO_2 from the dry matter, the nutrient concentrations were identical between the treatments. Therefore, the quantity of the nutrients in the bamboo parts is the same between treatments, but the concentration decreased because of the contribution of the precipitated Si to the dry matter. This result is consistent with the similar uptake of nutrients that was observed between the Si treatments.

Increasing the Si concentration in the nutrient solution did not significantly modify the growth parameters: our results did not reveal any positive effect on the numbers of leaves and stems, the height of the stems or the total biomass. Si has often been described in the literature to promote plant growth. Indeed, Hattori et al. (2003) showed that supplementation with 1.67 mM Si in hydroponic culture enhanced sorghum root elongation, and Hossain et al. (2002) reported that Si promoted the seedling growth of several *Poaceae* species, namely rice, oat and wheat, by increasing cell-wall extensibility. Dakora et al. (2003) reported that cowpea root growth increased significantly in hydroponic culture when Si was added. In our study, neither stem growth nor total root weight was affected by Si, and root growth was not measured; this is in contrast to some horticultural crops, such as cucumber, courgette and rose, for which the total weight increased following the application of potassium silicate (Hwang et al. 2005; Voogt et al. 2001). In most cases, however, it is uncertain whether the growth stimulation was attributable to a nutritional effect or to the alleviation of biotic and abiotic stresses. Indeed, the misinterpretation of the results of some experiments have been described by Marschner et al.

(1990). For example, Miyake and Takahashi (1978) (tomato), Miyake and Takahashi (1983) (cucumber), Miyake and Takahashi (1985) (soybean) and Miyake and Takahashi (1986) (strawberry) argued that Si was essential for plant growth because they observed that the lack of Si in the nutrient solution caused severe leaf chlorosis, depressions in flower and fruit formation, malformation and wilting. However, Marschner et al. (1990) demonstrated that in these experiments, the toxicity symptoms were caused by a nutrient imbalance, which is characterised by a high P concentration and a low Zn concentration; they also demonstrated that the Si effect was indirect because it increased the physiological availability of Zn, which thereby reduced its deficiency.

Several studies have also reported that Si fertilisation in field trials may increase and sustain the productivity of rice, sugarcane and non-accumulator species, such as tomato and strawberry (Korndorfer et al. 2001). Tavakkoli et al. (2011) found a significant increase in rice yield in a red Ferrosol following calcium silicate fertilisation. However, in field studies, it is likely that Si may increase yield by alleviating biotic and abiotic stresses, such as insect damage and lodging (Ma and Yamaji 2006). In hydroponic culture, Fauteux et al. (2006) demonstrated that Si alone did not affect *Arabidopsis thaliana* metabolism. In some other *Poaceae* plants, high levels of Si did not affect wheat growth (Rafi and Epstein 1999), banana growth, the rate of water and nutrient uptake (Henriet et al. 2006) or *Brachiaria brizantha* forage production (de Melo et al. 2010). One could therefore speculate that Si indirectly benefits plant growth by alleviating stress.

In this study, it is noteworthy that the bamboo in the Si0 treatment developed normally and did not display any deficiency symptoms. Because the Si content in the plants that were treated with Si0 were below the detection limit (<5 mg g^{-1}), it seems that the requirement of bamboo for Si is low. This should be investigated in further studies that can assess the requirement of Si as a mineral element for bamboo; for example, the content of Si in the solution could be decreased, and the Si content in bamboo could be quantified with a more sensitive technique (Guntzer et al. 2010). Indeed, despite the results of several studies that have described the effect of Si, its requirement for plant growth is still debated (Fauteux et al. 2006).

4.2 Effects of copper on bamboo growth

On average, the leaves and stems contained 16.6 ± 3.6 mg kg^{-1} and 9.8 ± 3.8 mg Cu kg^{-1} DW. These concentrations were higher than those of several bamboo species that were grown in non-contaminated soil; these plants contained 3.5 and 4.5 mg Cu kg^{-1} DW in the stem base and tip, respectively, and 5.1 mg Cu kg^{-1} DW in the leaf (Collin et al. 2011), which indicates that bamboo

responded to Cu in solution. However, the leaf concentrations were still within the range that is considered to be normal for mature leaf tissues (5-30 mg kg^{-1} Cu) (Kabata-Pendias and Mukherjee 2007). With an average of 131 ± 39.3 mg kg^{-1}, more than 80 % of the Cu was concentrated in the roots of the bamboo plants. This pattern is consistent with a number of studies that indicate that Cu accumulates more in plant roots than in the shoots or leaves (Ali et al. 2002; Ouzounidou et al. 1995). Because we did not desorb Cu from the roots, we cannot distinguish between adsorption and absorption by the roots. However, a high proportion of Cu in the roots may be bound to the cell wall (Nishizono et al. 1987). Indeed, cell walls are rich in compounds that can bind Cu, such as pectin polysaccharide and glycoprotein constituents (Krzeslowska 2011). It is likely that the rapid decrease in the nutrient solution during the first hours may be explained by Cu adsorption onto the root surface. We observed this rapid decline throughout phase II; it occurred following each contact with the bamboo plants, which may indicate that the Cu-binding capacity of the roots was not saturated even at the end of the experiment. Bravin et al. (2010) suggested that the Cu-binding capacity of the root apoplasm can reach approximately 1 g Cu kg^{-1} root DW. In the present study, the quantity of Cu that was delivered to the plant may represent approximately 0.4 g Cu kg^{-1} root DW per plant (data not shown). Thus, the binding capacity of the bamboo roots was certainly not entirely saturated with Cu. Due to its ability to bind Cu^{2+} onto its roots, the use of bamboo has been proposed for the adsorbent removal of Cu in wastewater (Babatunde et al. 2009).

Regardless of the Si treatments, no toxicity symptoms (i.e., leaf chlorosis, visible root changes or biomass decrease) were detected with a supply of 1.5 µM Cu^{2+} during phase II, although similar concentrations have been found to be toxic in *Poaceae*. Several authors have observed chlorosis in durum wheat seedlings at Cu^{2+} solution levels that were above either 0.55 µM (Bravin et al. 2010) or 1 µM (Michaud et al., 2008); a 50 % reduction of the Sabi grass fresh shoot biomass at 1.2 µM Cu^{2+} and the fresh root weight at 1.0 µM Cu^{2+} (Kopittke et al. 2009); and a 50 % reduction in root elongation in durum wheat at a Cu^{2+} solution concentration of either 0.06 µM (Bravin et al. 2010) or 0.6 µM (Michaud et al. 2008). Root damage was observed in the roots of Rhodes grass that was grown with a solution concentration of 0.22 µM Cu (Sheldon and Menzies 2005). Moreover, a recent review of the literature over the past 34 years (Kopittke et al. 2010) reported that the median toxic Cu concentration was 2 µM in hydroponic studies.

Several hypotheses may explain the absence of toxicity symptoms in our experiments. First, this result may be due to a modification of the Cu concentration and speciation in the solution. The initial concentration was effectively 1.5 µM Cu^{2+}; this was confirmed by both chemical modelling and Cu-ISE. Once it was in contact with the plant, the Cu content in the solution decreased abruptly in the first hours and stabilised to a value between 0.72 and 0.91 µM. After 24 h, the Cu

concentration increased slightly and homogeneously until the next renewal, but this increase may be explained by the decrease in volume of the nutrient solution due to plant uptake and evaporation. The ISE results that were combined with the calculations with PHREEQC using the composition and pH of the output solution indicate that the remaining Cu is primarily present as free copper. Therefore, the Cu^{2+} content in the solution was smaller than in the input solution; however, when compared to the work of Bravin et al. (2010) and Sheldon and Menzies (2005), the Cu^{2+} in the solution should have been sufficient to induce toxicity symptoms in durum wheat.

Second, the absence of any observed toxicity may be a consequence of the high biomass that was produced during phase I. Typically, and in the previous cited studies, toxicity measurements were performed on 2- or 3-week-old plants; these young plants have a lower biomass than the plants that were used in the current study (Kopittke et al. 2010). In our case, once the Cu was absorbed and translocated, it may have been diluted in the large number of stems and leaves, which may have induced an improved Cu tolerance through a lower internal Cu concentration. This hypothesis can be ruled out because in our experiment, the Cu concentrations in the bamboo leaves were 16.6 mg Cu kg^{-1}, while they reached 21 mg Cu kg^{-1} in the experiments that were performed by Michaud et al. (2008) and Kopittke et al. (2009) when the plants were exposed to a similar Cu content (1.1-1.2 µM Cu^{2+}).

Finally, the main differences between the previous studies and our study are the exposure length (2 months vs. 8 to 10 days) and the plant maturity (mature plants vs. seedlings). The large root biomass might may have contributed to tolerance through a Cu dilution in/onto the roots. In contrast to the shoots, the Cu content in the bamboo roots (an average of 131 ± 39.3 mg Cu kg^{-1} DW) was lower than the concentrations that were measured in the roots of the aforementioned studies (approximately 350 mg Cu kg^{-1} DW in durum wheat (Michaud et al. 2008) and 890 mg Cu kg^{-1} DW in Sabi Grass (Kopittke et al. 2009)). The binding of Cu onto the cell wall reduces the amount of Cu that is internalised; if the binding sites are not saturated, the roots may be better able to prevent translocation to the stems and leaves, which therefore protects the most sensitive sites from Cu toxicity. It has been suggested that the sequestration of trace metals in cell walls is one of the main strategies that plants use to cope with metal stress (Krzeslowska 2011). In durum wheat, Michaud et al. (2008) suggested that a root Cu content of approximately 100-150 mg kg^{-1} is the critical level that corresponds to early and moderate Cu phytotoxicity. In comparison, the Cu content in the bamboo roots is within the lower range of the Cu concentration that may induce toxicity.

Based on its high biomass and its adsorption capacity, the bamboo *Gigantocloa sp.* "Malay Dwarf" tolerates high Cu concentrations in nutrient solution.

4.3 Effects of bamboo Si on Cu sensitivity

Despite the addition of Cu into the nutrient solution, no differences regarding the biomass measurements or the Cu content in plants were observed between the Si treatments. These results contrast with those of the study of Nowakowski and Nowakowska (1997), which showed that the addition of 0.4 mmol L^{-1} Si significantly decreased the Cu content in the roots and in shoots of 7-day-old wheat seedlings. Two other studies also demonstrated the Si alleviation of Cu toxicity in *Arabidopsis thaliana* (Brassicaceaea) (Khandekar and Leisner 2011; Li et al. 2008) and *Erica andevalensis* (Ericaceaea) (Oliva et al. 2011). In both studies, the Si supplementation induced an increase in the shoot biomass and a reduction of chlorosis symptoms. However, the mechanisms by which Si alleviates Cu toxicity are still poorly understood. Li et al. (2008) demonstrated that supplemental Si did not change the total Cu concentrations in the leaf tissue, suggesting that Si influenced the distribution or bioavailability of Cu within the leaves (Rogalla and Romheld 2002). Conversely, Oliva et al. (2011) observed that Si induced a significant reduction of the Cu concentration in the leaves, which likely occurred by inhibiting the translocation of Cu from the roots to the shoots. Interestingly, Li (2008) and Khandekar and Leisner (2011) observed that Si altered gene expression. In response to high Cu, Si significantly decreased the expression of two *Arabidopsis* copper transport genes, copper transporter 1 (COPT1) and heavy metal ATPase subunit 5 (HMA5). Si helps to reduce stress through a more efficient response to Cu toxicity by maintaining or up-regulating Cu-binding molecules and by increasing the expression of free-radical-metabolising enzymes (Khandekar and Leisner 2011).

The quantities of Cu that were used in these studies were higher than the concentrations that were chosen in the present study; Oliva et al. used 500 µM (2011), and Khandekar and Leisner (2011) and Li et al. (2008) used 30 µM. However, in *Erica* plants, even at the lowest Cu treatment (1 µM), Si significantly decreased the Cu content, whereas in the present study, the Cu content was not affected by Si supplementation.

In our study, we identified two different mechanisms that regulate the uptake of Si and Cu. Si is absorbed homogeneously between two renewals, whereas Cu is primarily adsorbed in the first hours after each solution renewal. As a result, Cu is mainly accumulated in the roots, and it has a low concentration in aerial parts, whereas Si was accumulated in high amounts in the leaves. These differences in the uptake and repartition of Cu and Si in the plant may indicate that the bamboo plant utilises two different strategies to govern the allocation of these elements. Therefore, differences in the response of bamboo plants when compared to *Arabidopsis* or *Erica* may be due to the differences in Cu and Si acquisition, transport or interaction in plants.

Copper uptake mechanisms in monocotyledonous species are poorly understood when compared to those in dicotyledonous species; the mechanisms in monocotyledons have primarily been studied in *Arabidopsis* (Yruela 2009). However, it is known that Si uptake mechanisms and their ability to accumulate Si differ between phylogenetic groups and plant species (Hodson et al. 2005; Ma and Yamaji 2006). Cu and Si in *Erica* are both accumulated in the roots (Oliva et al. 2011), whereas in bamboo, Si is primarily accumulated in the leaves. The Si concentration varied widely between different plant families; for example, the leaf Si content ranged from 0.565 to 1.2 mg g^{-1} in *Erica*, ranged from 0.084 to 0.566 mg g^{-1} in *Arabidopsis*, and ranged from below the detection limit to 218 mg g^{-1} in bamboo (our study). Therefore, the plant response to the Si supply is certainly different between Si-accumulating and non-accumulating plants.

Our data lead us to conclude that Si displayed no significant effects when it was supplied to plants that were growing under controlled conditions, although the bamboo Gigantocloa sp. "Malay Dwarf" did absorb Si in large amounts. The different absorption mechanisms for Cu and Si may partially explain why Si did not influence the Cu repartition and concentration in bamboo. No toxicity symptoms were observed for the applied Cu concentration, which were relevant to Cu concentrations that have been measured in contaminated soil; this indicates that bamboo plants are tolerant to Cu. Therefore, it seems that bamboo can cope with large Cu soil concentrations and could thus be used in phytoremediation schemes. However, this result should be further investigated by testing higher Cu concentrations, and it should be confirmed with field experiments.

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5 BILAN DU CHAPITRE III

Malgré des temps de culture longs, la **culture hydroponique** s'est révélée adaptée à la croissance des bambous sympodiaux, et nous a permis de suivre avec précision l'évolution des nutriments dans la solution nutritive.

L'accumulation de Si dans le bambou *Gigantochloa* sp *«* Malay Dwarf *»* est proportionnelle à la concentration de Si en solution. Dans le traitement de Si le plus élevé, la concentration en Si des feuilles est de 218 ± 19 mg g^{-1} en moyenne. En revanche, **Si n'a pas eu d'influence sur le développement et la croissance des bambous**.

Le suivi des concentrations de Si et Cu dans la solution nutritive nous a permis d'identifier **deux mécanismes d'absorption différents** pour ces deux éléments dans la plante : une absorption progressive de Si, et une adsorption rapide et importante de Cu sur les racines.

L'accumulation de Si dans les différentes parties de la plante suit la tendance suivante : feuilles > tiges > racines, alors que la distribution de Cu est la suivante : racines > tiges ≥ feuilles. Les concentrations de Cu ne sont pas modifiées par les différents traitements de Si.

Grâce à sa forte biomasse racinaire, et à sa capacité à séquestrer Cu dans les racines, **le bambou a toléré la forte concentration de Cu** à laquelle il a été exposé.

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CHAPITRE IV

COPPER DISTRIBUTION AND SPECIATION IN BAMBOOS "*Phyllostachys fastuosa*" EXPOSED TO DIFFERENT COPPER AND SILICON CONCENTRATIONS

TABLE DES MATIERES

CHAPITRE IV

COPPER DISTRIBUTION AND SPECIATION IN BAMBOO "*Phyllostachys fastuosa*" EXPOSED TO DIFFERENT COPPER AND SILICON CONCENTRATIONS

Dans des conditions non ou peu stressantes, le silicium n'a pas eu d'effets sur la croissance et le développement du bambou sympodial *Gigantocloa sp.* « Malay Dwarf » malgré la forte accumulation de Si dans ses tissus. Cela est cohérent avec des observations effectuées pour d'autres plantes, comme le bananier (Henriet et al. 2006) ou des plantules de conifères (Cornelis et al. 2010) : le silicium jouerait un rôle lorsque la plante est soumise à un stress biotique ou abiotique (Liang et al. 2007). Or, il s'avère que la concentration de Cu choisie dans l'étude précédente n'a pas induit de toxicité chez le bambou, contrairement à ce qui a été observé pour d'autres *Poaceae* telles que le blé et la Sabi grass (Bravin et al. 2010; Kopittke et al. 2009; Michaud et al. 2008). Nous n'avons donc pas pu, dans cette étude, statuer sur l'effet de Si face à une toxicité de Cu. En revanche, nous avons identifié deux comportements différents de l'absorption au sens large, de Cu et Si : alors que Si est prélevé de manière continue au cours du temps, Cu est prélevé de manière rapide les premières heures après chaque renouvellement de la solution nutritive. Cela semble indiquer une adsorption rapide de Cu sur les parois racinaires qu'il serait intéressant de vérifier par une observation précise de la localisation du cuivre dans la racine en tentant d'estimer la proportion de Cu adsorbée.

La toxicité du cuivre dépend non seulement de sa localisation dans les différents tissus mais aussi de sa spéciation. La plante adopte différentes stratégies pour gérer un excès de cuivre dans ses tissus, comme une séquestration dans les parois cellulaires (Krzeslowska 2011), ou bien une complexation par différents ligands (Cf chapitre I). Un des mécanismes permettant d'expliquer la réduction d'une toxicité grâce à Si est la formation de complexes métaux-Si, comme cela a été proposé pour Zn dans *Cardaminopsis halleri* (Neumann and zur Nieden 2001). L'étude de la spéciation de Cu par spectroscopie d'absorption des rayons X doit nous permettre d'identifier la

nature des complexes formés avec Cu, d'éventuelles interactions entre Cu et Si et/ou une modification de la spéciation de Cu induite par Si telle qu'une modification des ligands.

Nous avons vu dans le chapitre II que, dans les conditions pédoclimatiques de l'île de la Réunion, les bambous sympodiaux accumulent significativement plus de Si et de Cu que les bambous monopodiaux. Les bambous monopodiaux, qui sont les plus répandus dans les pays tempérés, présentent-ils également la capacité de tolérer une concentration forte de Cu (1.5 µM Cu^{2+}) en solution ? C'est ce que nous avons testé dans cette expérience conduite en hydroponie avec le bambou *Phyllostachys fastuosa* et deux niveaux de concentrations en Cu : 1.5 et 100 µM Cu^{2+}. Cette dernière concentration a été choisie afin d'induire une forte toxicité et d'observer l'effet de Si sur cette toxicité.

Les objectifs de ce chapitre sont les suivants :

- Evaluer la distribution et la localisation de Cu et Si dans les différentes parties du bambou,
- Evaluer l'effet de Si sur la concentration et la toxicité de Cu,
- Evaluer l'effet de Si et Cu sur la nutrition (acides organiques et inorganiques) du bambou,
- Caractériser la spéciation de Cu dans le bambou.

Pour répondre à ces objectifs, plusieurs techniques ont été utilisées : en premier lieu des analyses chimiques des macro et micronutriments, puis l'identification et la quantification de composés organiques et inorganiques par chromatographie ionique, ensuite la localisation de Si et Cu dans les racines par microscope électronique et micro-fluorescence X, et enfin la spéciation de Cu par spectroscopie d'absorption X en couplant le XANES (X-ray Absorption Near Edge Structure) et l'EXAFS (Extended X-ray Absorption Fine Structure). Les résultats sont présentés dans l'article ci-après, qui a été rédigé afin d'être soumis à la revue *New Phytologist.*

Copper distribution and speciation in bamboo "*Phyllostachys fastuosa*" exposed to different copper and silicon concentrations. First evidence of the presence of reduced copper bound to sulfur compound in a *Poaceae* species.

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SUMMARY

- The purpose of this study was to examine copper (Cu) absorption, repartition and toxicity and determine the role of a Si supplementation in a *poaceae* species: the bamboo *Phyllostachys fastuosa.*
- An hydroponic culture was carried out for 3 months, bamboos were submitted to three contrasted copper concentrations, and two silicon (Si) concentrations. Copper speciation and Cu and Si distribution were investigated by chemical analyses, spectroscopic and microscopic techniques .

- Copper is mainly accumulated in bamboo roots, where a large part of Cu is retained in epidermis. In roots, silicon is mainly accumulated in endodermal cells. In roots, stem and leaves, Cu was present under two oxidation states Cu(I) and Cu(II) with different proportions.
- Silicon supplementation modified the Cu speciation but did not induced significant improve of bamboo Cu tolerance. The main strategy of bamboo to cope with high Cu concentration in its tissues is (i) an important sequestration in the roots apoplast, mainly in epidermis, and (ii) a Cu(I) complexation with sulfur ligands both organic and inorganic.

Keywords: toxicity, alleviation, Extended X-ray absorption fine structure spectroscopy (EXAFS), X-ray Absorption Near-Edge Spectroscopy (XANES), microfluorescence X (μ-XRF)

1. INTRODUCTION

Copper plays a central role in plant metabolism but is detrimental when critical thresholds are exceeded. Excess copper is cytotoxic, induces stress and causes injury to plants. Cu contamination is widespread as a result of its use in fungicides, application of pig slurries, atmospheric deposition from industrial and urban activities and metalliferous mining. Phytoremediation of trace metals has been proposed for wastewater treatment. Bamboos are known for their resistance to a wide range of stress factors as well as their high growth rate and biomass production. Bamboos are also used in phytoremediation technologies (Arfi et al. 2009). Therefore in order to optimize this technology it is essential to test the extent of Cu tolerance in bamboos and thereafter to identify mechanisms used by bamboos to cope with an excess Cu content. Speciation of Cu associated to its distribution within the plant ultimately determines the various mechanisms of availability, detoxification and toxicity (Lombi et al. 2011). X-ray absorption spectroscopy (XAS) is a powerful tool to assess the chemical speciation in plants (Salt et al. 2002) and can be used to determine the oxidation sate and the physical structure of sites surrounding Cu in plant tissues. Cu speciation had been reported in the following plants; *Larrea tridentate* (Gardea-Torresdey et al. 2001; Polette et al. 2000), *Vigna unguiculata* (Kopittke et al. 2011), *Sesbania drummondii* (Sahi et al. 2007), *Elsholtzia splendens* (Shi et al. 2008), *Thlaspi caerulescens* (Mijovilovich et al. 2009), *Crassula helmsii* (Kupper et al. 2009). However, no studies have been performed on monocotyledonous species, and particularly *poaceae* family. *Poaceae* plants, and bamboos in particular, are able to accumulate high amounts of silicon (Si) in their tissues (Collin et al. 2011). Recently, it has been shown that silicon alleviates Cu toxicity in *Arabidopsis thaliana* (Khandekar and Leisner 2011; Li et al. 2008) and in *Erica andevalensis* (Oliva et al. 2011). However, we showed in a previous study, that a wide range of Si in solution did not influence the growth and development of bamboos *Gigantocloa sp* "Malay dwarf" which were submitted to a Cu concentration of 1.5µM Cu^{2+} in hydroponics (Collin et al, chap III). This Cu concentration did not induce toxicity symptoms in bamboos contrary to effects described in other *Poaceae* plants such as wheat (Bravin et al. 2010) and Sabi grass (Kopittke et al. 2009) exposed at similar Cu content. Silicon is known to be beneficial mostly under abiotic or biotic stress (Liang et al. 2007). The fact that Collin et al. (chap III) did not observe the effect of Si on bamboo may be related to the absence of stress conditions. To confirm that, it is necessary to test Si with a higher Cu concentration, that may induce toxicity in bamboos.

The previous hydroponic culture of bamboos (Collin et al, chap III) were done on a monopodial type of bamboo, which has been shown to accumulate significantly more Cu and Si than sympodial bamboos in natural pedo-climatic environment (Collin et al. 2011). We therefore can wonder if the ability to tolerate high Cu content depends on the bamboo species. Thus we also chose to test Cu tolerance on a sympodial bamboo: *Phyllostachys fastuosa.* Bamboos were grown in a 4-month hydroponic experiment and submitted to the same Cu content (1.5 µM) as in (Collin et al, chap III) which is large but environmentally relevant (Sauvé et al. 1997), and to a higher Cu concentration in order to reach acute toxicity and test the possible Cu alleviating effect of Si. In order to identify the possible Cu tolerance mechanisms in bamboos supplemented or not with Si, we investigated the *in situ* distribution and speciation of Cu using several combined approaches: mineral and organic analyses, Cu and Si localization in the roots investigated by X-ray fluorescence micro-spectroscopy (µXRF) and scanning electron microscopy coupled to energy dispersive X-ray analysis (SEM-EDX), Cu speciation determined by Cu K-edge Extended X-ray absorption fine structure spectroscopy (EXAFS) and X-ray Absorption Near-Edge Spectroscopy (XANES).

2. MATERIAL AND METHODS

2.1. Plant material, experimental design and pre-culture

Thirty-five one-year old monopodial bamboos *Phyllostachys Fastuosa* grown on the same substrate were provided by PHYTOREM (Miramas, France). Bamboos were transferred in hydroponic culture after a careful washing of the roots in order to remove all the soil particles. A preliminary experiment was carried out to adapt hydroponic culture to bamboos (Collin et al, chap III). The experiment was performed during 4 months. A pre-culture phase was conducted over 2 months. Plants were grown in cylindrical PVC pots containing 3 L of nutrient solution. The composition of the pre-culture nutrient solution was a 25 % strength complete Hoagland solution. The composition in macroelements (mM) was: 1 $Ca(NO_3)_2$, 1.5 KNO_3, 0.24 $MgSO_4$, 0.22 $(NH_4)_2HPO_4$, and in microelements (µM): 11.6 H_3BO_3, 0.08 $CuSO_4$, 0.2 $ZnSO_4$, 0.03 MoO_3, 2.3 $MnCl_2$, 100 FeNaEDTA. The pH of this solution was adjusted to 6 with NaOH or HNO_3. The nutrient solutions were continuously aerated with an air pump in each pot. The nutrient solution was renewed every 7 days. Analytical-grade chemical reagents and ultra pure water were used in the preparation of nutrients solutions. No glassware was used to minimize Si contamination. All plastic ware used for the experiments were previously soaked overnight in nitric acid and rinsed with ultrapure water. The growth chamber parameters were set at (day/night): 28/25°C, 90/75% of relative humidity and 450 µmol photons m^{-2} s^{-1} light intensity.

2.2. Silicon and copper treatment

At the end of the pre-culture phase, 25 bamboo plants of uniform size were selected. Five treatments were applied during 70 days, 2 concentrations of Si and 3 concentrations of Cu with the following combinations: 0 mM Si + 0.1 µM Cu, 0 mM Si + 1,5 µM Cu, 1.1 mM Si + 1,5 µM Cu, 0 mM Si + 100 µM Cu, 1.1 µM Si + 100 µM Cu thereafter referred to respectively as control, Cu1.5, Cu1.5Si, Cu100, Cu100Si. Si was provided as monosilic acid from potassium-metasilicate $Si(KOH)_2$, which has a K:Si mole ratio of 2 (Metso 400 - YARA) (Voogt et al. 2001). Cu was added using $CuSO_4$. Potassium and hydroxide levels were adjusted in the nutrient solution to compensate for the additional input of K and OH from the silicon addition. KNO_3 and HNO_3 concentrations were adjusted in each treatment to reach K= NO_3 = 2.25 mM. The composition of macroelements (mM) in this nutrient solution was 1 $CaCl_2$, 0.24 $MgSO_4$, 0.22 $(NH_4)_2HPO_4$ and the composition of microelements (µM) was: 11.6 H_3BO_3, 0.2 $ZnSO_4$, 0.03 MoO_3, 6.5 $MnCl_2$. Fe was provided as 20 µM Fe-N, N9-di (2-hydroxybenzyl) ethylenediamine-N, N9-diacetic acid monohydrochloride hydrate (HBED; Strem chemical, USA). Fe(III)-HBED was prepared following the description of Chaney et al. (1998) in such a way that all HBED was saturated with Fe. Solution pH was set at 6.0 (±0.2) buffered with 1 mM MES (2-morpholinoethanesulphonic acid). Five replicates per treatment were conducted. For each treatment, the nutrient solution circulated continuously between the five pots and the 15L "reserve tank". The solution was continuously supplied by a peristaltic pump from the reserve tank to the base of the pot and the solution in excess of 2.5 l was released and collected via an overflow pipe to come back in the reserve tank. The nutrient solutions were continuously aerated with an air pump in each pot. All solutions contained in each pot and in the reserve tank were totally renewed every 7 days.

Geochemical calculations were performed with PHREEQC (version 2-17) (Parkhurst 1995) and the MINTEQ database of thermodynamic constant. The quality of speciation modelling was checked by measuring Cu^{2+} with a Cupric Ion Selective Electrode (Orion 9629BNWP). To measure Cu in treatment Cu1.5 and Cu1.5Si, Cu-ISE measurements were calibrated between pCu^{2+} 12.0-4.2 in 10^{-4}M Cu solution buffered with iminodiacetic acid and potassium phtalate according to the procedure described by Rachou et al. (2007). Due to the high Cu concentration of treatment Cu100 and Cu100Si, ISE measurements were calibrated between 30-500 µM using a direct measurement in Cu standards.

Throughout the experiment, nutritive solution was sampled at the beginning of the week (before contact with bamboos) further referred to as "input solution" and just before the renewal, further referred as "output solution". All the solutions were filtered (0.2 µm membrane, Sartorius) and kept at a temperature of 4°C temperature. The pH in each solution was measured

with a combined glass-electrode. A subsample was acidified and solutions were analysed for Si by ICP-AES (Jobin Yvon J38), and total Cu concentration was measured by ICP-MS (Agilent 7500 CE, Montpellier). Free Cu^{2+} was analysed by Cu-ISE in another subsample, immediately after solution sampling. The Cu-ISE measurement was conducted on the following selected samples: for treatment Cu100 and Cu100Si, in all the input and output solutions and for treatment Cu1.5, Cu1.5Si: in the input solution on day 196 and in the solutions that were sampled after 1, 2, 4, 27, 43, and 96 h; in the input solution on day 27 and at day 28 and 29 (after one and two day of solution contact with plant); and in the output solution that was collected on day 34.

The total volumes of each input and output solution were measured. The volume of the input solution was 14,5 L on average, and output volume varied between 9,2 and 5 L. The amount of element (Si or Cu) in the solution is the element concentration measured by ICP-AES or ICP-MS multiplied by the volume of the solution. Errors calculated from the errors on output volume of solutions and analytical errors are estimated to be 15 %.

2.3. Plant analysis

2.3.1 Growth parameters

The number and height of the stems, the number of leaves and the fresh weight were assayed at the beginning of the experiment (day 0), during the experiment (day 21 and day 49), and at the end of the experiment (day 226).

2.3.2 Analysis of macro and micronutrients

At the end of the experiment, the 25 plants were harvested. Plant leaves, stems, rhizomes and roots were separated. The samples were carefully washed with ultrapure water and the fresh masses were determined.

Before the analysis, all of the parts were dried at 60°C until they reached a constant weight. The samples were subsequently mixed, ground and homogenised. The sub-samples were dried at 80 % until they reached a constant weight to determine their dry weight. The plant samples (leaves, stems and roots) underwent dry mineralisation for the analysis of trace elements. During the mineralisation, the Si content was determined by gravimetry as follows: 500 mg of dried plant material was placed in a platinum dish and gradually heated to 500°C. The silica was eliminated in the ash with HF. After cooling, the Si weight was determined based on the difference from the 500°C weight. The ashes were dissolved into HCl, and the content of trace

elements in the solutions was analysed by inductively coupled plasma-optical spectrometer (ICP-OES Vista-Pro, Varian, CIRAD, Montpellier, France). For quality control, in-house reference samples and certified samples (Astrasol-Mix, Analytika) were used every 20 samples, and each analysis was conducted in duplicate. The measurement uncertainty was less than 15 %. The quantification limit for Si was 5 mg g^{-1} of dry weight (DW).

The concentrations of macro- and micronutrients are expressed as g kg^{-1} or mg kg^{-1} DW, and the Si concentrations are expressed in mg g^{-1} DW SiO_2 such that the results are directly comparable with the literature.

2.4. Desorption procedure

A set of frozen root sub-samples was used to measure the adsorbed Cu after the extraction of apoplasmic Cu with HCl on thawn roots. The extraction procedure was previously detailed and tested by Chaignon et al. (2002). Briefly, a subsample of 0.4 g of fresh (thawed) roots was shaken end-over-end with 20 ml of 1 mM HCl for 3 min, then 180 µl of 1MHCl were added to yield a final concentration of 10 mM HCl. After shaking for a further 5 min, the suspensions were filtered through an ashless filter paper (Whatman 40). Copper in the suspensions were analysed by ICP-MS (MSE, Montpellier). Root samples were then rinsed 3 times with ultrapure water, a part of the sample were dry at 80°C in order to measure the dry weight and the other part was frozen in liquid nitrogen to study copper speciation: these roots samples were further referred as "root desorbed". Copper adsorbed on the roots were calculated and expressed in µg.kg^{-1} DW from the quantity of Cu in the suspensions. The root desorbed concentrations were calculated as total Cu concentration in roots minus Cu adsorbed concentration.

2.5. Anion assays

Plant material were freeze-dried and ground in a blender (Retsh, MM301). Approximately 10 mg of resulting powder were immersed in 1 ml of 50 % (v/v) methanol at 4°C, then 300 µL chloroform was added. The samples were shaken for 20 min, 40 rpm at 4 ° C and centrifuged at 13200 rpm, 4°C, for 10 min. 800 µL of upper phase was collected and evaporated to dryness using a centrifugal vacuum dryer at ambient temperature for 4 h and redissolved in 1600 µL of ultra-pure water. Anions concentrations were determined by High Performance Ionic Chromatography (Dionex DX 600) using the IonPaq AS11HC anion exchange column and a NaOH gradient. NaOH concentration was raised linearly from 1.25 to 25.0 mM over 8 min, then from 25.0 to 65.0 mM over 45 min. Identification and quantification of each anion were performed by

comparison of the retention times and peak areas – integrated using Chromeleon software (Dionex) – with the standards.

2.6. Total soluble amino acid

Frozen plant materials were homogenised with a pestle and mortar in liquid nitrogen. An aliquot of 1 mg of the homogenate was mixed in 10 ml of 0.1M and centrifuged at 12500 rpm, 4°C for 10 min. The upper phase were collected, filtered at 0.22 µm and immediately analyzed for amino acid determination. Amino acids were detected by High Performance Ionic Chromatography (Dionex ICS3000) using a trap column (CRC) and an amino acid column (AminoPac PA10) with a NaOH gradient. We used a volume injection of 20µM and a flow rate of 0.25mL/min. Detection was performed with Au working electrodes (060082 Dionex). Identification and quantification of asparagine, threonine, glycine, histidine, cysteine were performed by comparison to standards *AAA-Direct* method (Dionex).

2.7. SEM-EDX

Two samples of roots of treatment Cu100Si were freeze-fractured in a cryo specimen chamber, sputter-coated with a mixture of gold and palladium in argon atmosphere, and examined using a Philips XL30 SFEG scanning electron microscope (SEM) at nitrogen liquid temperature (North Billerica, MA) coupled to an Oxford Instruments (Oxfordshire, UK) energy dispersive X-ray spectrometer (EDX). The SEM was operated at 15 kV with a counting time of 60 s per point. With SEM-EDS, chemical microanalysis results can be obtained with a spatial resolution of about 2 to 5 µm and a penetration depth of about the same range.

2.8. Laboratory-Based µXRF

Root samples were dried at 40°C, embedded in an epoxy resin and cut transversely with diamond wire saw. The thickness of the obtained sections was of 200 µm. The measurements were carried out on a HORIBA XGT^{7000} microscope equipped with an X-ray guide tube producing a finely focused and high-intensity beam with a 10-µm spot size (Rh X-ray tube, accelerating voltage of 30 kV and a current of 1mA). X-rays emitted from the irradiated sample were detected with an energy-dispersive X-ray (EDX) spectrometer equipped with a liquid nitrogen cooled high-purity Si detector. Elemental maps (256 px^2, pixel size of 8 mm), showing in particular Cu, Si and K distribution in root cross sections, were recorded with a total counting time of 20*1000s under total vacuum mode (to enhance Si detection). The background contribution was

removed for Si and Cu. Images were obtained from the intensity of the Si(Kα), K(Kα) and Cu(Kα) emission lines.

2.9. EXAFS and XANES: data acquisition and analysis

Copper K-edge bulk X ray absorption spectra for the plant leaves, stems, roots and reference compounds were recorded on a FAME Beamline at the European Synchrotron Radiation Facility (ESRF, Grenoble, France). In order to avoid artifactual changes of speciation due to the drying of samples, the samples were plunged in liquid nitrogen immediately after the harvest and plant washing, and stored frozen. Frozen leaves, stems and roots were ground and compacted into pressed pellets in liquid nitrogen (77 K), with special care to keep the pellets frozen in liquid nitrogen until the XAS analysis. Pellets of frozen-hydrated plant samples and reference compounds were transferred to a He cryostat and cooled to 15 K. Spectra were recorded in fluorescence mode using a Si(220) double crystal monochromator and a 30-element solid-state Ge detector (Canberra, France). For each sample, 1 to 14 scans of 45 min each were averaged. To reduce the risk of beam damage and to obtain representative spectra, each scan was done on different position in each specimen. The energy was calibrated using a Cu foil (threshold energy taken at the zero-crossing point of the second derivative spectrum). The data were normalized using the Athena software (Ravel and Newville 2005). EXAFS spectra were Fourier transformed from the *k* to *R* space using Kaiser–Bessel apodization Windows. This procedure results in pseudo-radial distribution functions (RDF) uncorrected from phase shift functions.

In this study, we used a combination of principal component analysis (PCA), target transformation (TT), and linear combination fitting (LCF) to fit Cu K edge XANES spectra (-30-50keV) and k^3-weighted EXAFS (2.6-10.5 $Å^{-1}$) recorded on the plants. The goodness of fit was assessed with the normalized sum-square (NSS) equation $NSS = 100 \times \left(\sum_{i=1}^{N} \left[k^3 \chi(k_i)_{measured} - k^3 \chi(k_i)_{fitted} \right]^2 \right) / \left(\sum_{i=1}^{N} \left[k^3 \chi(k_i)_{measured} \right]^2 \right)$, N is the number of points, $k^3\chi(k_i)_{measured}$ is the EXAFS spectrum of the sample in the k-space and $k^3\chi(k_i)_{fitted}$ is the EXAFS fit in the k-space). PCA and TT were done with the SixPack software and LCF was done with Athena software. This procedure has already been used to fit XANES spectra that contain multiple chemical species (Beauchemin et al. 2002; Legros et al. 2010). Reference compounds are chosen from a large panel of potential chemical species that could contribute to explaining the coordination, oxidation state, and atomic environment of Cu within plant samples. Cu K-edge XANES spectra and k^3-weighted EXAFS spectra for Cu model compounds, contained 10 inorganic and 9 organic compounds with various Cu oxidation states (Cu(0), Cu(I), and Cu(II)) were

recorded (listed in SI I).For further details about this XANES and EXAFS analysis See SI II and SI III.

2.10. Statistical analyses

The Minitab 15.1 software package (Minitab, Inc.) was used for statistical analyses. For each part of the plants, concentrations of micro and macronutrients studied were analysed by ANOVA. We used a one-way ANOVA at the 95 % confidence level with Si treatments (5 levels) as the main factor, followed by LSD's post hoc test at the 95 % confidence level to evaluate differences in treatments. Average concentrations of Si and Cu in root, stem and leaf were compared using paired t-Test at the 95 % confidence level.

3. RESULTS

3.1. Silicon and copper in nutrient solution

During the contact with bamboos, i.e. during 7 days, amounts of Si and Cu in the nutrient solution decreased (Figure IV.1). Cu amount decreased sharply during the first days in both Cu treatments and then decreased slowly during the last 6 days. Total Cu measured in solution by ICP-MS was well correlated to pCu measured by ISE (R^2=0.88) (data not shown). The addition of Si in nutrient solution did not change Cu concentration in solution (data not shown). The decrease of Si amount in the nutrient solution was smaller in Cu100Si treatment than in Cu1.5Si (Figure IV.1). Throughout the experiment and in all treatments, pH slightly increased after the contact with plants, with a final pH varying between 6 and 6.8.

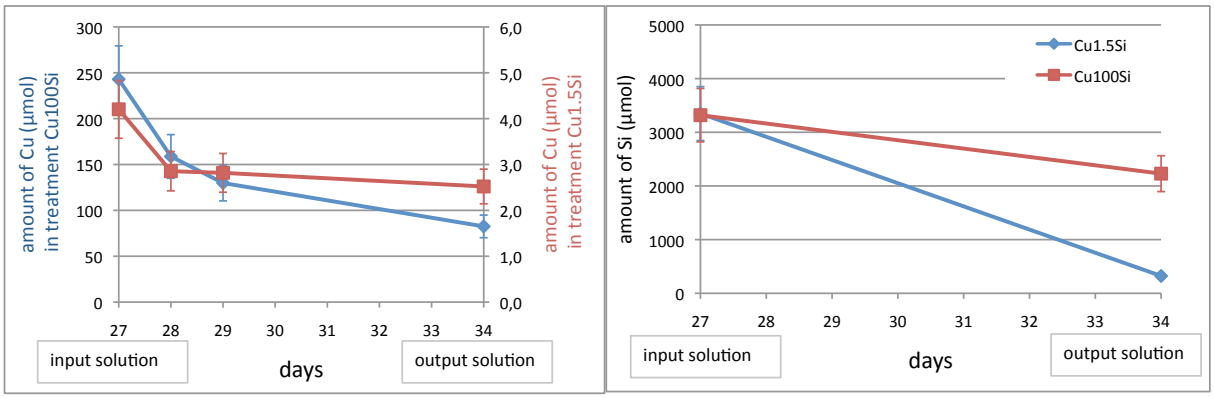


Figure IV.1 Si and Cu amount (µmol) in the nutrient solution in treatment Cu1.5Si and Cu100Si from day 27 to 34

3.2. Growth parameters

During the first 22 days, the numbers of leaves and stems developed in all treatments were not significantly different, ranging from 86 ± 31 to 169 ± 103 leaves per bamboos (Figure IV.2). After that period, the average number of leaves in treatment Cu100 and Cu100Si decreased and reached an average number of 31±23 and 48±52 leaves per bamboo at the end of the experiment, which is significantly different from the 230 ± 52 leaves in the control. Throughout the experiment, all the growth measurements were statistically similar from treatments Control, Cu1.5 and Cu1.5Si (data not shown). Significant differences between mean numbers of stems and fresh masses were also measured between treatments Cu100, Cu100Si on one hand and treatments Cu1.5, Cu1.5Si, control on the other hand. Therefore the 100 µM Cu concentration in solution induced a significant growth reduction contrary to the 1.5 µM Cu concentration. This toxicity was confirmed by visual symptoms: chlorotic leaves and brown coloration of roots, which was less pronounced in Cu100 Si treatment than in Cu100 treatment. (Figure IV.3). The presence of Si in nutritive solution increased the leaves number for Cu100Si treatment as compared to Cu100 treatment but this increase was not significant (Figure IV.2).

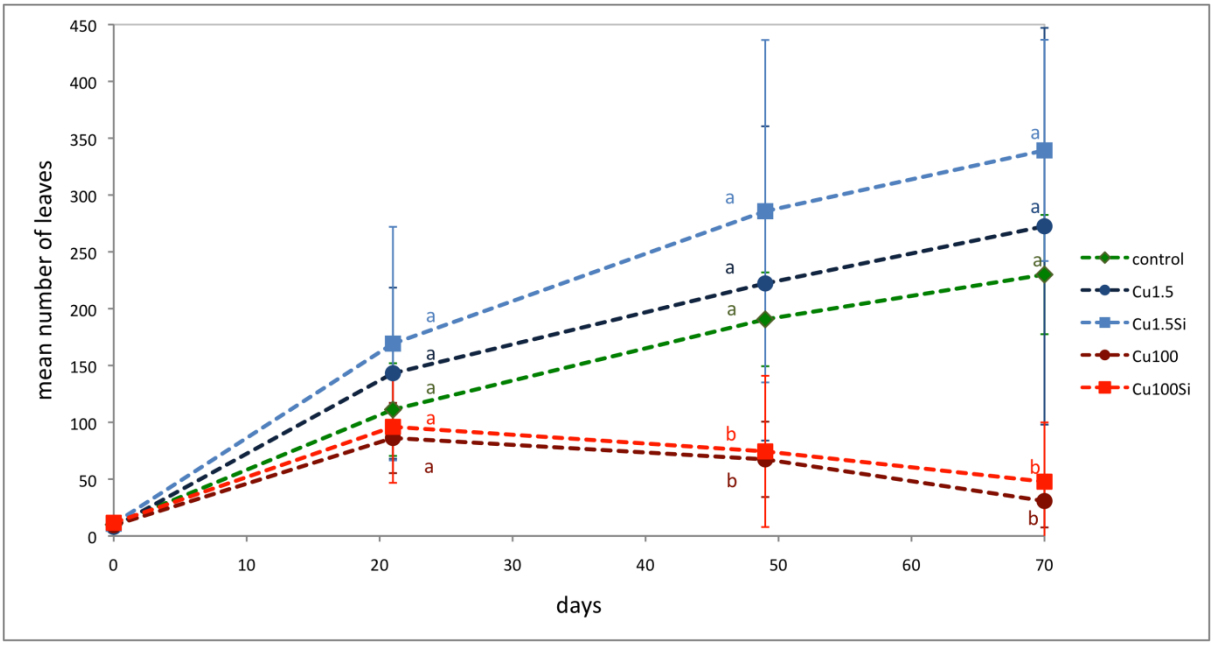


Figure IV.2 **Mean number of bamboo leaves in the 5 treatments, throughout the experiment from day 0 to day 70. Values followed by same letter for the same days are statistically not different according to Tukey test at the 95 % confidence level.**

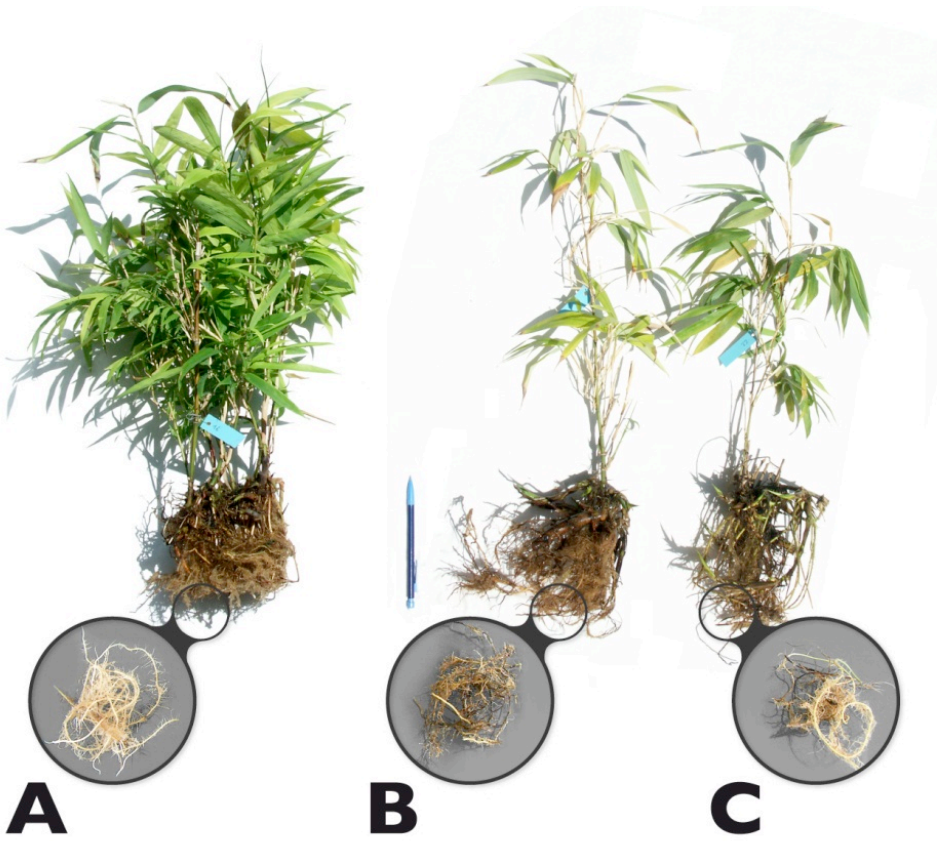


Figure IV.3 **Photographs of whole plants harvested after 70 days in treatments: (A) control; (B) Cu100; (C) Cu100 Si**

3.3. Cu and Si distribution between plant parts

Table IV.1 shows the average Cu and Si concentrations and their statistical significance between different parts of bamboos, whereas Figure IV.4 compares concentrations between treatments. Cu concentrations in all bamboo parts in treatments Cu1.5, Cu1.5Si and control were similar (Figure IV.4). The Cu concentrations in plant tissues, both leaves, stems and roots, are significantly higher in treatments Cu100 and Cu100Si than in other treatments. Copper concentrations in aerial parts of bamboo were not affected by Si in both Cu treatments. Differences between root total Cu content in treatment Cu1.5 and Cu1.5 Si were not significant due to high individual variability of Cu content (147 ±145 for Cu1.5 treatment) (Figure IV.4). The adsorbed Cu concentration was significantly higher in treatment Cu100Si (2409±1263 mg g^{-1} DW) than in treatment Cu100 (1333±327 mg g^{-1} DW) (Figure IV.4), but concentrations in root desorbed were not different. The adsorbed copper concentration represented 41.5 % and 61.5 % of total root Cu concentration in treatment Cu100 and Cu100Si. In all treatments, Cu accumulatio followed the order root>stem≥leaf, with 70 to 96 % of Cu retained in the roots (Table IV.1). Roots concentration (total Cu) ranged from 14.7 to 3915 mg g^{-1} DW whereas leaves

concentration ranged from 2.4±0.1 to 35.9±8 mg g^{-1} DW. Silicon accumulation followed the order leaf>stem>root. Copper supply decreased significantly Si concentration in leaves with an average of 92.8±31.3 mg Si g^{-1} in treatment Cu1.5Si, and 42 ±11.8 mg.g^{-1} in treatment Cu100Si.

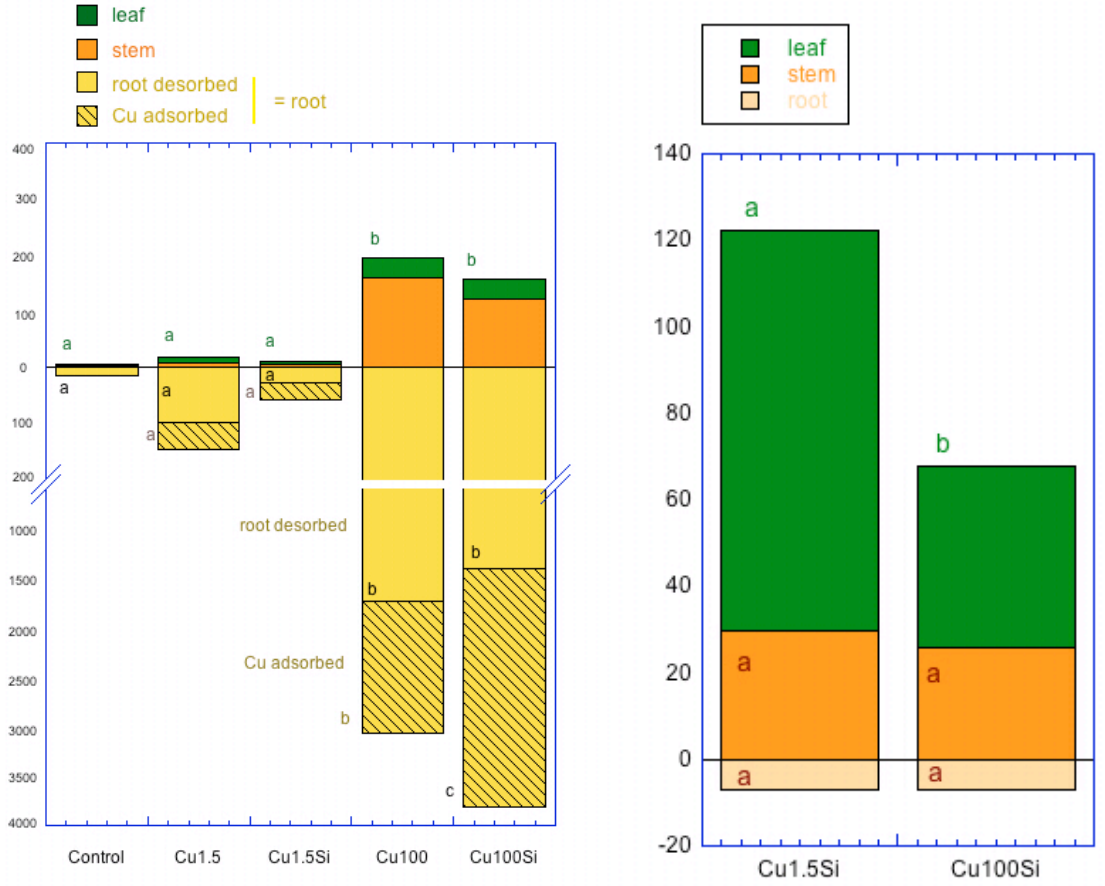


Figure IV.4 **Cu and Si concentration in leaves, stems, roots in the different treatments. Values followed by same letter between treatments are statistically not different according to Tukey test at the 95 % confidence level.**

Table IV.I Cu and Si concentrations in plant parts of bamboos grown in the five treatments (n=4, mean ± standard deviation)

treatments	plant parts	Cu (mg kg^{-1})		Si (mg g^{-1})	
Control	leaves	2.4 ± 0.1	c	<QL	
	stems	3.7 ± 0.4	b	<QL	
	roots	14.7 ± 5.2	a	<QL	
Cu1.5	leaves	9.9 ± 0.5	a	<QL	
	stems	8.5 ± 3.0	a	<QL	
	roots	147 ± 145	a	<QL	
Cu1.5Si	leaves	5.4 ± 2.0	b	92.8 ± 31.3	a
	stems	4.5 ± 3.0	b	29.7 ± 7.6	b
	roots	58.1 ± 24.8	a	5.4 ± 2.0	c
Cu100	leaves	35.9 ± 8.0	c	<QL	
	stems	161 ± 54.5	b	<QL	
	roots	3171 ± 1223	a	<QL	
Cu100Si	leaves	35.5 ± 5.8	c	42.0 ± 11.8	a
	stems	122 ± 56.9	b	25.9 ± 9.9	b
	roots	3915 ± 653	a	6.5 ± 2.4	c

Values followed by same letter within each treatment are statistically not different according to Tukey test at the 95 % confidence level. <QL: under the quantification limit of 5 mg g^{-1}

3.4. Cu and Si localization in the root

The examination of a root cross section belonging to the treatment Cu100Si was performed with SEM which is a powerful imaging technique because of its nanometer-scale spatial resolution and allows the individual 'spot' analysis. This technique was chosen to compare concentrations in different tissues of the roots (i.e epidermis, cortex, endodermis, stele)(Figure IV.5). EDX spectra were then recorded to determine their elemental composition. Silicon was detected in different cells but mostly in the endodermis cells. Due to the low detection limit of Cu with SEM-EDX, Cu was only weakly detected in epidermal cells (Figure IV.6). In order to better localize these elements, another root cross section was examined with µXRF, which has a better detection limit for Cu and allows for 2D imaging of elemental distributions. The µXRF mapping confirmed the MEB observations: Si was detected mostly in endodermal cells, whereas Cu was detected mainly in epidermal cells.

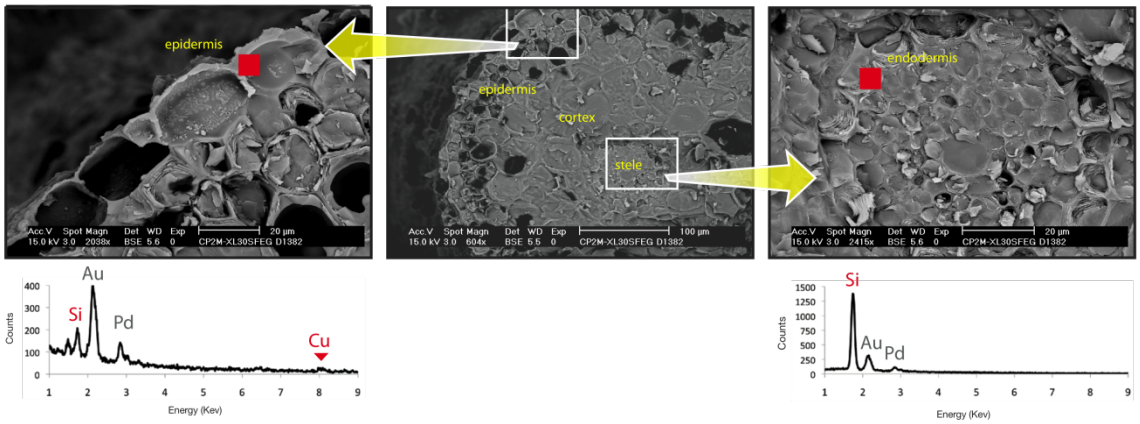


Figure IV.5 **cryo-SEM image with two EDX spectra taken in the root epidermis cells (A) and in the endodermis cells (B)**

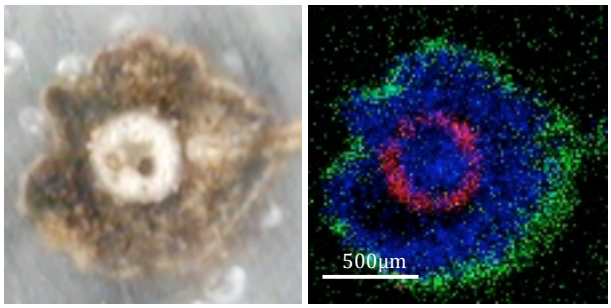


Figure IV.6 **µXRF image of a root cross-section from treatment Cu100Si, tricolour image combining Si (red), K (blue) and Cu (green).**

3.5. The effects of copper and silicon on some plant components

The 100 µM Cu treatment decreased significantly the concentrations of the macro and micronutrients N, Ca, Mg, Mn in leaves and K, Mg in roots (Table IV.2). The addition of Si increased Ca content in roots and decreased Zn content in stems. The content of P and Fe in leaves and roots were not significantly modified by Cu and Si treatments. The content of inorganic acids, carboxylic acids and amino acids are presented in Table IV.3. Among the inorganic acid, nitrate and phosphate concentrations in roots were significantly decreased by Cu both in treatment Cu1.5 and Cu100, with or without Si. Sulfate concentration in roots were significantly smaller in Cu100 and Cu100Si treatment than in other treatments. Sulfate concentration in leaves were decreased in all the Cu treatments as compared to the control,

sulfate concentration in stems was also reduced but it was not significantly. Chlorure content was not modified whatever the treatments.

Few changes were observed for the carboxilic and amino acids: measured between treatments. Cu in treatment Cu100 and Cu100Si induced a significant decrease in citrate and malate contents in roots and in isocitrate content in leaves. The only significant modification of amino acid was observed for histidine concentration in roots and, contrary to other elements, an increase was observed in treatment Cu100 (0.78 $mmol.kg^{-1}$) and Cu100Si (0.88 $mmol.kg^{-1}$) as compared to the control (0.34 $mmol.kg^{-1}$) (Table IV.3).

Table IV.2 **N, P, K, Ca, Mg, Fe, Mn and Zn concentrations in leaves and roots of bamboos from control, Cu100 and Cu100Si treatments (n=4, mean ± standard deviation)**

		N	P	K	Ca	Mg	Fe	Mn	Zn
		g kg^{-1}					*mg kg^{-1}*		
Leaf	Control	59.8 ± 10 a	3.5 ± 0,5 a	24.3 ± 4.3 a	10.4 ± 1.7 a	4.1 ± 0.6 a	57.2 ± 22.8 a	462 ± 133 a	27 ± 4.8 a
	Cu100	25.2 ± 1.1 b	3.7 ± 1,1 a	25.0 ± 1.0 a	4.5 ± 2.1 b	1.5 ± 0.3 b	60.9 ± 20.4 a	129 ± 54 b	17 ± 8.7 ab
	Cu100Si	25.5 ± 1.6 b	5.1 ± 1,5 a	27.3 ± 5.6 a	4.4 ± 2.5 b	1.9 ± 0.4 b	62.2 ± 15.7 a	148 ± 79 b	13 ± 4.9 b
Root	Control	19.9 ± 2.6 a	4.6 ± 0,7 a	21.0 ± 2.1 a	0.8 ± 0.2 ab	0.9 ± 0.2 a	227 ± 80.8 a	102 ± 56 a	11 ± 2.4 a
	Cu100	17.5 ± 4.2 a	1.9 ± 0,8 a	10.7 ± 3.6 b	0.7 ± 0.2 b	0.4 ± 0.0 b	469 ± 90.2 a	48.3 ± 5.8 a	17 ± 6.8 a
	Cu100Si	19.2 ± 1.0 a	2.2 ± 0,1 a	10.5 ± 2.2 b	1.1 ± 0.2 a	0.6 ± 0.2 b	469 ± 201 a	68.4 ± 14 a	16 ± 3.3 a

Values followed by same letter within plant parts for each element are statistically not different according to Tukey test at the 95% confidence level

Table IV.3 **Composition of inorganic acids, carboxylic acids and amino acid in leaves, stems and roots of plant grown in treatments control, Cu100 and Cu100Si (mmoles kg dry weight^{-1}, n=4 for inorganic acids and carboxylic acids, n=3 for amino acids). The standard deviation was not indicated for the lisiblity of the table.**

			inorganic acid				carboxilic acids				amino acids				
(mmol.kg^{-1}) DW		dry matter (%)	sulfate	chlorure	nitrate	phosphate	malate	oxalate	citrate	isocitrate	asparagine	threonine	glyine	histidine	cysteine
leaf	Control	32.8 a	32.9 a	227 a	4.1 a	74 a	13 a	59 a	28 a	48 a	0.97 a	1.39 a	5.79 a	0.57 a	7.52 a
	Cu1.5	34.4 a	21.2 b	223 a	4.9 a	54 a	15 a	59 a	23 a	41 ab					
	Cu1.5Si	40.7 a	16.7 b	163 a	4.9 a	41 a	15 a	89 a	17 a	23 b					
	Cu100	46.9 a	10.1 b	210 a	9.3 a	84 a	33 a	53 a	12 a	14 b	0.77 a	3.17 a	2.09 b	0.67 a	1.43 a
	Cu100Si	39.7 a	11.4 b	211 a	7.8 a	88 a	18 a	44 a	14 a	30 b	1.39 a	2.82 a	2.72 b	0.83 a	3.74 a
stem	Control	47.3 a	12.8 ab	63 a	17 a	72 a	17 a	5.6 a	32 a	6.1 a	0.86 a	0.75 a	2.99 a	0.61 a	0.19 a
	Cu1.5	45.8 a	18.4 a	85 a	18 a	90 a	17 a	12 a	29 a	5.5 a					
	Cu1.5Si	49.1 a	10.2 bc	64 a	19 a	48 a	19 a	7.9 a	23 a	3.8 a					
	Cu100	64.1 a	8.6 bc	62 a	17 a	62 a	20 a	3.9 a	20 a	4.4 a	1.04 a	0.49 a	1.10 a	0.64 a	0.33 a
	Cu100Si	60.1 a	4.4 c	51 a	14 a	48 a	8.2 a	2.8 a	30 a	5.7 a	1.85 a	0.19 a	1.01 a	0.79 a	0.26 a
root	Control	16.9 a	27.9 a	143 a	155 a	122 a	19 a	2.4 a	16 a	1.2 a	1.87 a	2.52 a	2.09 a	0.34 a	18.9 a
	Cu1.5	23.9 a	26.3 a	108 a	105 ab	61 b	12 b	1.0 a	9.2 bc	1.0 a					
	Cu1.5Si	24.8 a	25.2 a	95 a	46 b	34 b	16 ab	0.7 a	13 ab	0.6 a					
	Cu100	30.4 a	5.5 b	123 a	47 b	23 b	4.8 c	0.9 a	5.3 cd	0.5 a	1.31 a	1.86 a	2.77 a	0.78 b	12.2 a
	Cu100Si	28.3 a	5.8 b	98 a	51 b	31 b	2.7 c	1.0 a	2.1 d	0.4 a	1.18 a	2.26 a	1.89 a	0.88 b	17.5 a

Values followed by same letter within plant parts for each element are statistically not different according to Tukey test at the 95% confidence level

3.6. XANES features

XANES spectra analysis was used to investigate the local atomic coordination surrounding Cu in different parts of bamboo plants. Several inflections (A, B, C, D), defined in XANES references spectra and peaks in the first derivative (Figure IV.7), provide information on the Cu oxidation state and the Cu symmetry compound (Legros et al. 2010). The Cu K-edge XANES spectra of plant samples differed between plant parts (Figure IV.7) suggesting that Cu speciation in roots, stems and leaves was different. When Cu is monovalent a well defined absorption feature (B) is located at 8982 eV which corresponds to the transitions 1s-$4p_{xy}$ for the Cu(I) compounds (Kau et al. 1987). This feature is well exhibited in leaf and stem samples of treatment Cu1.5 and Cu100 in both Si treatments, indicating the presence of Cu(I). In desorbed roots samples, the peak B in the first derivative allows us to identify also Cu(I) but at a weaker intensity than in leaf or stem samples (Figure IV.8). The pre-edge feature (peak A) corresponding to dipole-forbidden electronic transitions 1s to 3d (quadrupole) is visible only in root and root-desorbed samples around 8977.8 eV. Feature C (8988 eV) and D (8995 eV) are the 1s to 4p main electron edge transitions of Cu(II) compound (Figure IV.8). These features correspond respectively to the 1s to $4p_z$ electron transition and 1s to $4p_{xy}$ (Furnare et al. 2005). The splitting of the main edge peak results from the anisotropic symmetry of Cu(II) compounds (Jahn-Taller distortion). The feature D is present in all plant samples, indicating that a fraction of Cu is divalent Cu. Roots samples with Si and without Si show the same XANES spectrum, which is close to an organic acid spectra such as malate (Figure IV.7). The splitting of the 4p orbital is 5.3 and 5.5 eV in root and desorbed root samples, corresponding to an axial Cu-O distance of approximately 2.35 Å as compared to about 1.95 Å for the equatorial distance (Figure IV.7) (Dupont et al. 2002; Manceau and Matynia 2010).

In order to evaluate the number of individual species, which reconstruct the whole set of XANES spectra (n=10), principal component analysis (PCA) was applied (See SI Table IV.6 and Table IV.7). A combination of two components was sufficient to provide satisfactory fits. The XANES spectra were best fitted with the reference compounds Cu(I)cysteine and Cu(II)malate or Cu(II)acetate, in different proportions depending on plant parts. The main differences between aerial parts and roots was the proportion of Cu(I) and Cu(II) (Figure IV.9 and SI Table IV.8). We observe that the proportion of Cu(I) in leaves, stems, and roots was higher in treatment Cu100Si than in treatment Cu100.

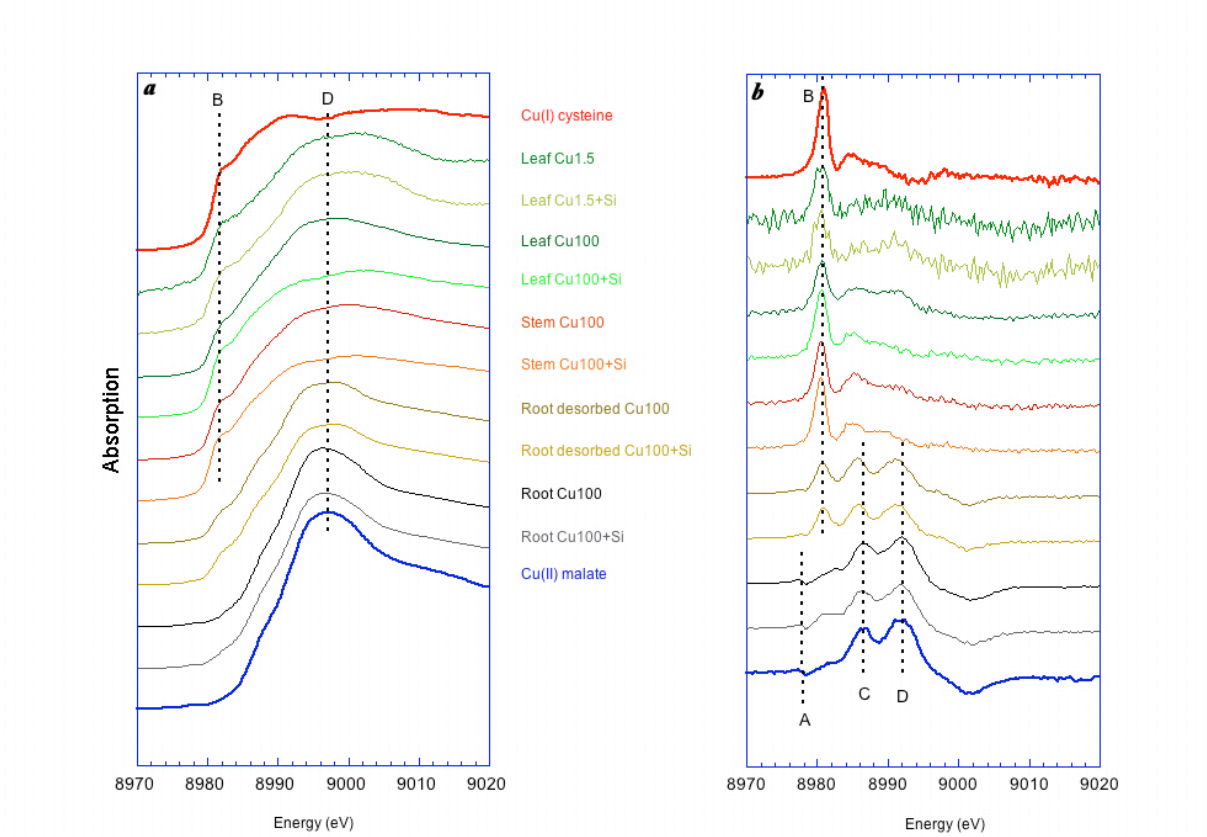


Figure IV.7 **(a) Normalized Cu K-edge XANES spectra of plant samples and (b) the corresponding first derivatives of Cu K-edge XANES spectra.**

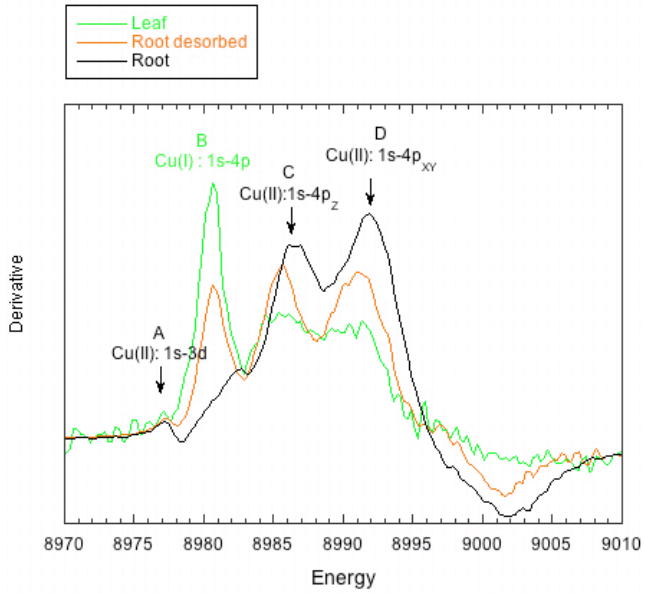


Figure IV.8 **The derivative of XANES spectra showing the evolution of the feature B in different part of the plant and the splitting of the 4p orbitals resulting from the Jahn-Teller distortion, in leaf Cu100, desorbed root Cu100 and root Cu100.**

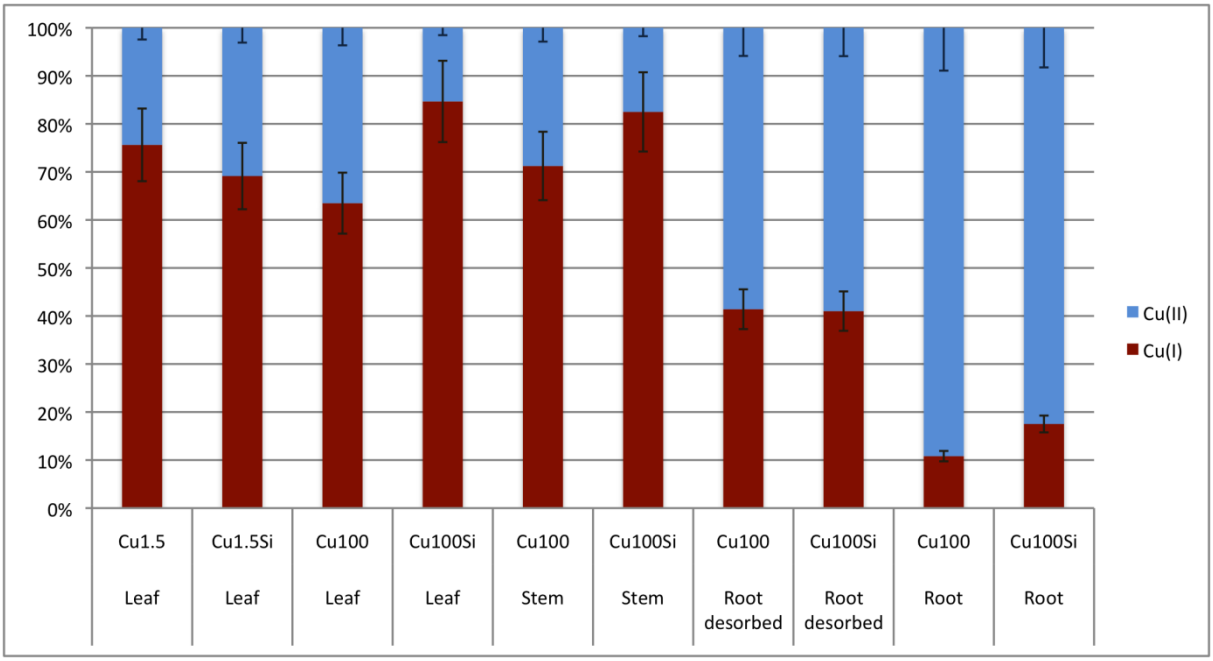


Figure IV.9 **Proportions of Cu(I) and Cu(II) determined by LCF on XANES spectra. The proportions were adjusted to reach 100 %.**

3.7. EXAFS features

To gain more insight into the molecular structure of Cu in plant, the EXAFS Spectra were compared (Figure IV.10) with two reference compounds that have coordination shells: Cu(I)-cysteine and Cu(II)-malate. For Cu(I)-cysteine, Cu was surrounded by S atoms located 2.34 Å from the central atom (Cu-S binding), whereas for Cu(II)-malate, Cu was bound to four O atoms at a distance ranging from 1.95 to 1.97 Å (Cu-O binding). This was reflected on the EXAFS spectra of these reference compounds by out-of-phase oscillations and by RDF peaks, for the first coordination shell, which was shifted to 1.9 Å for Cu(I)-cysteine (feature D) and to 1.4 Å for Cu(II)-malate (feature C) (distances uncorrected for phase shifts). The leaf and stem EXAFS spectra (Figure IV.10) showed oscillations similar to Cu(I)-cysteine at 6.2 and 7.7 $Å^{-1}$ (feature A), whereas roots spectra showed oscillations similar to Cu(II)-malate at 6.5 and 8.5 $Å^{-1}$ (feature B). Comparing the EXAFS spectra, we detected a shift from a dominant Cu-O binding pattern (root) to a dominant Cu-S binding pattern (leaves). This pattern was clearly expressed by the RDF pattern. The peak at 1.4 Å (uncorrected for phase shifts) was dominant in roots and its amplitude gradually decreased in the aerial part of bamboos, whereas the intensity of the peak at 1.9 Å (uncorrected for phase shifts) increased. Therefore EXAFS spectra confirm that different types of ligands are involved in metal complexation in various parts of the plant.

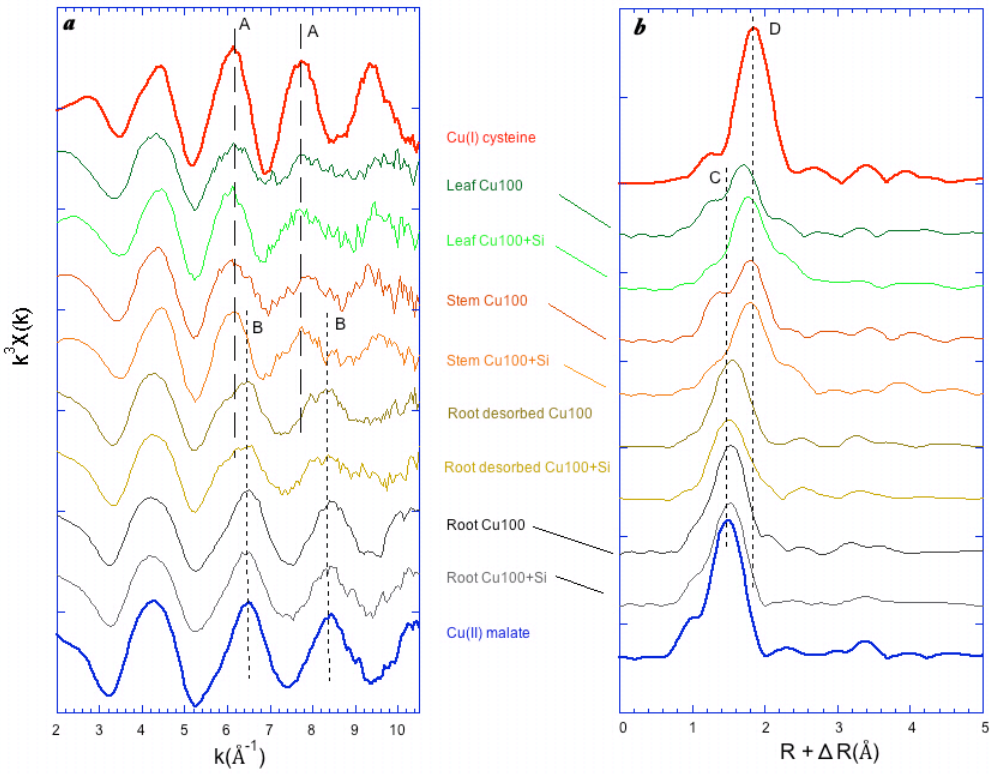


Figure IV.10 **(a) Cu K-edge extended X-ray absorption fine structure spectra; (b) radial distribution function (RDF) for plant samples. k = photoelectron wave number ($Å^{-1}$), R = distance to the neighboring atom (Å).**

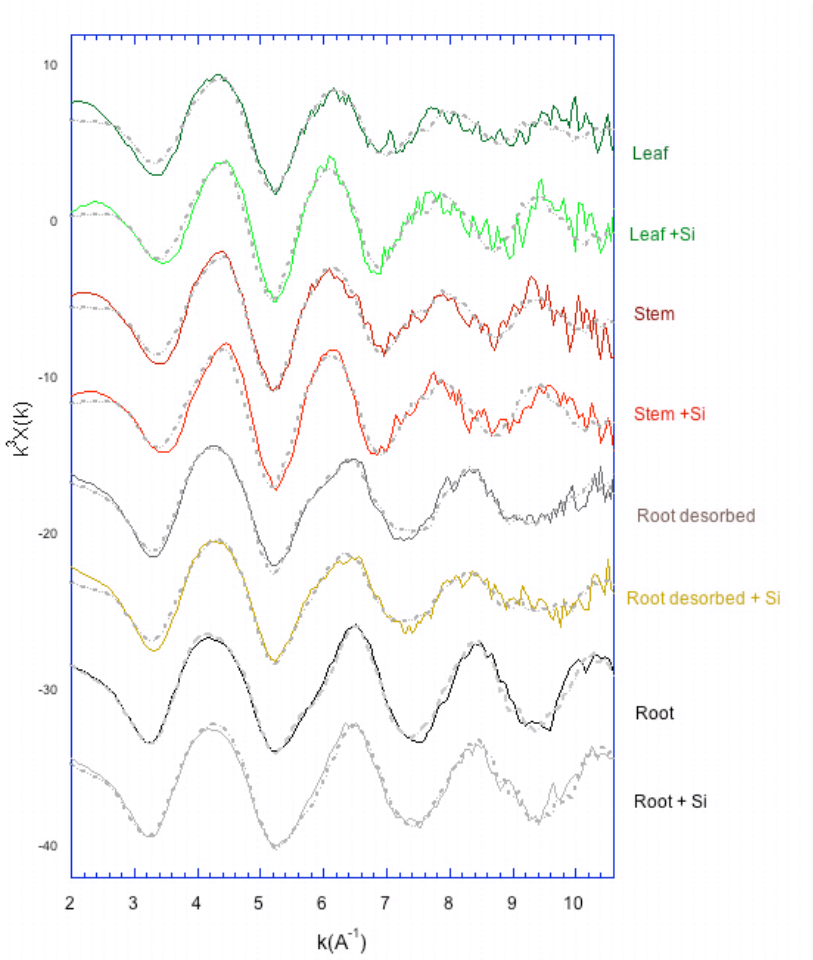


Figure IV.11 Cu K-edge k^3-weighted EXAFS (solid line) and linear combination fits (dotted line) for plant samples.

The proportions of the Cu species in the eight samples were determined by LCF upon the model compounds suggested. In roots, best component fits were obtained with 50 % Cu(II)-malate, 50 % Cu(II)-histidine for the Cu100 treatment and with 100 % Cu(II)-malate for the Cu100Si treatment (Figure IV.11 and Table IV.10). Malate was used as a representative of Cu(II) binding to alcohol groups and carboxyl groups, and histidine as of a Cu(II) binding to amino N and carboxyl COOH groups. Manceau et Matynia (2010) showed that Cu in these two complexes is bound to two 5-(O/N)-rings. It is worth noting that oxygen can not be distinguished from nitrogen by EXAFS. This is due to their similar atomic number and to the fact that the distance between O and Cu in Cu-O bounding is usually very similar to the distance between N and Cu in Cu-N bounding. However, the LCF of Cu100 was significantly improved by the introduction of Cu-histidine reference, this may indicate a contribution of amine groups in Cu complexation for the Cu100 treatment and not for the Cu100Si treatment.

In desorbed roots of the Cu100 and Cu100Si treatments, approximately 20 % of Cu(I)-cysteine was detected, and the remaining proportion shows the same ligands than roots of the Cu100 treatment (Cu(II)-histidine and Cu(II)-malate) and Cu100Si (Cu(II)-malate) (Table IV.4). LCF analysis revealed that Cu in leaves and stems was predominantly associated with sulfur ligands (Cu_2S and Cu(I)cysteine) and the remaining fraction associated with an oxygen or nitrogen containing organic acid (Cu(II)-malate) (Table IV.4 Figure IV.13). The presence of both Cu(I)-sulphur ligands, i.e. Cu_2S : Cu(I)S-inorganic and Cu(I)-cysteine: Cu(I)S organic, could be questioned. But attempts to model leaf and stem samples with Cu_2S contributions always decreased NSS significantly (>30 %). For example, in leaf of treatment Cu100Si, adding Cu(I)S inorganic as third component improved the spectral match and decrease the NSS by 33 %, the best statistical agreement being obtained with a mixture of 33% Cu(I)-cysteine, 43 % Cu_2S, 24 % Cu(II)-malate (Figure IV.12). In stem samples Cu(II)-malate improved the LCF quality compared to Cu(II)-histidine (NSS decrease from 12.1 % to 9.8 %), but in leaves the LCF was similar with Cu(II)-histidine and Cu(II)-malate.

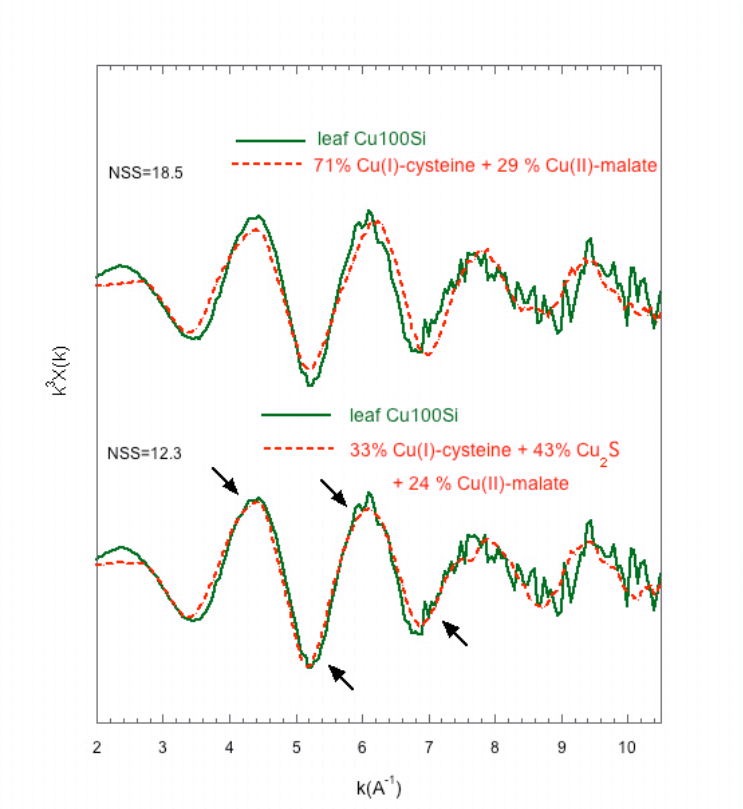


Figure IV.12 **Cu K-edge k3-weighted EXAFS spectra of leaf in treatment Cu100Si (solid line) and fits (dotted line) with and without the contribution of Cu2S compound (Cu(I)S-inorganic).**

Table IV.4 **Proportion of Cu species determined by LCF of the Cu K-edge EXAFS spectra. The proportions were adjusted to reach 100 %.**

		Cu_2S	Cu(I)-cysteine	Cu(II)-malate	Cu(II)-histidine	Sum	NSS (%)
leaf	Cu100	31	26	44		100	14.2
	Cu100Si	43	33	24		100	12.3
stem	Cu100	36	30	34		100	9.8
	Cu100Si	36	37	27		100	9.3
root desorbed	Cu100		23	38	39	100	4.1
	Cu100Si		26	74		100	6.9
root	Cu100			50	50	100	3.2
	Cu100Si			100		100	3.9

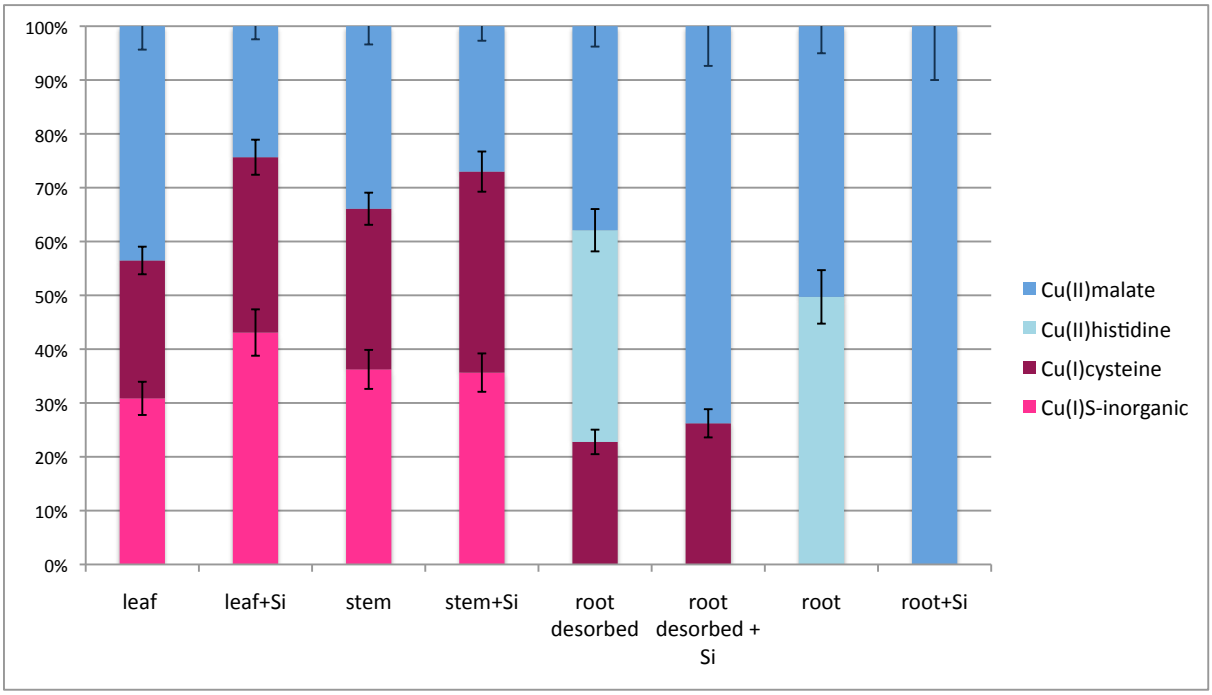


Figure IV.13 **Proportions of Cu compounds determined by LCF on EXAFS spectra. The proportions were adjusted to reach 100 %.**

The proportion of Cu(I) and Cu(II) measured by XANES and EXAFS are closed, and they show the same tendency between samples, i.e, an increase of Cu(I) proportions in Cu100Si treatment compared Cu100. Moreover, the best XANES and EXAFS LCF were done with the same reference compounds. The accordance between XANES and EXAFS results shows the robustness of the analysis.

4. DISCUSSION

The bamboo *Phyllostachys fastuosa* exposed to a Cu concentration of 1.5 µM Cu^{2+} in hydroponics did not express any toxicity symptoms. This result is in accordance with the tolerance of the bamboo *Gigantocloa sp.* "Malay dwarf" exposed to the same Cu content in a previous hydroponic study (Collin et al., Chap III). Therefore, monopodial bamboos *Phyllostachys fastuosa* and sympodial bamboos *Gigantocloa sp.* "Malay dwarf" seem to have the same ability to tolerate copper. This non-toxicity differs from observed toxicity in hydroponic culture of durum wheat (Bravin et al. 2010) and Sabi Grass (Kopittke et al. 2009) for similar Cu contents. Cu concentration in leaves, stems and roots in control and treatment Cu1.5 and Cu1.5 Si were not significantly different and the means ranged from 2.4 mg kg^{-1} in leaves to 147 mg kg^{-1} in roots (Table IV.1), which is closer to the Cu concentration measured in *Gigantocloa sp.* "Malay dwarf" exposed to 1.5 µM: 16.6 mg kg^{-1} in leaves and 131 mg kg^{-1} in roots (Collin et al. Chap III). Cu content in leaves and stems are closed to those of several bamboo species in a non-contaminated soil: 3.5 and 4.5 in stem base and tip and 5.1 mg kg^{-1} in leaf (Collin et al. 2011). With the addition of 100µM Cu^{2+} in solution, Cu content in leaves had an average of 35.9 and 35.5 mg kg^{-1} and a root average of 3171 and 3915 mg kg^{-1} in the treatment Cu100 and Cu100Si respectively (Table IV.1), which is the highest Cu content reported so far in bamboo plants (Collin et al. 2011, Collin et al, chap III). These concentrations are above the toxic thresholds of 20-30 mg kg^{-1} DW proposed by Marschner (1995) and above the critical root concentration of 250-300 mg.kg^{-1} measured in durum wheat (Michaud et al. 2008). Whatever the Si supplementation, we observed strong phytotoxicity symptoms as evidenced by the biomass decrease (Figure IV.2). In both Cu treatments, Si supplementation did not significantly modify the absorption of Cu whereas Si concentrations in bamboos parts were increased. However, visual symptoms (chlorosis and brown coloration of roots) were reduced by the Si supplementation. The measured Si concentration in leaves of *Phyllostachys fastuosa* (average of 92.8 mg.g^{-1}, Table IV.1) was smaller than Si concentration measured in a previous hydroponic study with the same Si content in solution for the bamboo *Gigantocloa sp* "Malay Dwarf" (179 mg.g^{-1}, Collin et al, chap III). Bamboos are known to accumulate Si throughout their live (Motomura et al. 2004), thus this difference may be explained by the smaller duration of this study (3 months) as compared to the previous 8 months study of Collin et al. (Chap III).

Toxic metal may interfere with essential nutrient uptake and transport, thereby disturbing mineral nutrition composition. In our study, Cu reduced the uptake of N, Ca, Mg, K, Mn and Zn (Table IV.2) in treatment Cu100 and Cu100Si. It is generally accepted that damage to the

plasmalemma of root cells constitutes the first effect of Cu toxicity by causing a loss of ions, such as K or other solutes (De Vos et al. 1991). The strong decrease of N concentration in leaves and NO^{3-} in roots (Table IV.2 and Table IV.3) shows that excessive Cu affected nitrogen and protein metabolism in plants. These observations are consistent with the decrease of NO_3^{2-}, amino acids and protein content induced by excess Cu in *E. haichowensis* (Li et al. 2007) and *in Silene vulgaris* (Weber et al. 1991).

The majority of Cu was stored or immobilized in bamboo roots, and only a little fraction (4 to 30 %) of total Cu was translocated from the root to aerial parts (Figure IV.4). The desorption procedure has shown that the fraction adsorbed, i.e. stored in the apoplast, represented 42 % and 61.5 % of total root Cu concentration in respectively treatments Cu100 and Cu100Si. Moreover, the examination of a root cross-section by SEM-EDX and µXRF, revealed that substantial deposition of Cu occurred in the epidermal region. This result is in agreement with several studies using different approaches: Cu analyses after isolating the cell walls (Konno et al. 2010; Lou et al. 2004; Nishizono et al. 1987), observation and analyses by SEM-EDX (Brunner et al. 2008; MacFarlane and Burchett 2000), localization using synchrotron-based X-ray fluorescence microscopy (Kopittke et al. 2011). In a previous hydroponic study with the bamboo *Gigantocloa sp* "Malay Dwarf", we observed a high and rapid decrease of Cu content in solution during the first hours after nutrient solution renewal. In this study we confirm the behaviour of Cu in solution, which decreased sharply during the first day in both Cu treatments. Therefore, the desorption procedure, the localization, and the evolution of Cu in the nutrient solution indicate the important role of Cu adsorption in bamboo roots. The ability of bamboo roots to bind Cu^{2+} has been suggested for a use as an adsorbent for the removal of Cu in wastewater (Babatunde et al. 2009). The cell walls of plants have the ability to bind copper on negatively charged sites, mainly on polysaccharide compounds rich in carboxyl groups (Krzeslowska 2011). The binding to plant cell walls may be a mechanism of heavy metal tolerance and a defensive strategy of plants to metal stress (Krzeslowska 2011; Nishizono et al. 1987).

The localisation of Si within root tissue, mainly in the region of endodermal cells, is in accordance with SEM observations in the bamboo *Phyllostachys heterocycla* (Lux et al. 2003) and Si deposition in other poaceae species (Hattori et al. 2003; Sparks et al. 2011). Endodermal cells are directly connected with the Casparian strips which provide selectivity against metal with reduced concentration entering the stele of root (MacFarlane and Burchett 2000). It is hypothesized that silica deposits in the endodermis cell wall along with the thickening of the Casparian strips is one of the mechanisms that reduce the metals apoplastic transport (da Cunha and do Nascimento 2009). This would be explained by a co-precipitation of Si and metal in the endoderm and by a reduction in the transpiration by-pass flow induced by the Si deposition (Shi

et al. 2005). In the present study, Cu was not detected in the endodermis and Cu concentration in aerial part was not affected by Si supplementation. Despite the important Si deposition in endodermis, the reduction of apoplastic by-pass of Cu and a co-precipitation does not seem to be a relevant mechanism here.

The quantity of adsorbed Cu is significantly higher in treatment Cu100Si than treatment Cu100. Another hypothesis to explain the alleviation of excess metal is the mediation of the metal binding to the cell wall, thereby lowering the symplasmic concentration while the total concentration was the same. This mechanism has been proposed for the Si alleviation of Mn toxicity in *Cucumis sativus* (Rogalla and Romheld 2002). More recently Li et al. (2008) suggested that Si decreases Cu toxicity by its sequestration in cell walls in *Arabidopsis thaliana*. A similar mechanism may occur in bamboo roots since Cu speciation in roots is influenced by the presence of Si in nutrient solution: Cu is bound to 50 % Cu(II)malate-like compounds and 50% Cu(II)histidine-like compounds in the treatment Cu100, whereas Cu is entirely bound to Cu(II)malate-like compounds in treatment Cu100Si (Table IV.3). In rice, Wang et al. (2004) suggested the formation of Al-Si complexes as a mechanism for the sequestration of Al into a non-phytotoxic form. But if Si seems to affect Cu speciation, Si was not detected in the local environment of Cu, contrary to what was suggested for Al-Si in rice roots (Wang et al. 2004).

XAS results on desorbed roots confirmed that a significant part of Cu in roots (≈40 % Figure IV.9) is present as Cu(I). We explained the fact that Cu(I) was higher in desorbed root compared to root, because its signature was diluted by the high amount of Cu(II) present in the adsorbed pool. As Cu is known to be a redox active metal in plants, the presence of the two oxidations state in plant tissue is in line with physiological studies (Yruela 2009). Copper may be reduced before its acquisition by root cells, or Cu may be reduced in the cytoplasm after acquisition, either chemically, metabolised enzymatically or by cell macro-molecules (Meharg 2005). It has been shown, in *Arabidospis thaliana,* that Cu is most likely to enter the cytosol of root cells via a cell surface COPT-family transporter (Sancenon et al. 2003). The COPT proteins transport Cu in a reduced form, so a reduction step may be necessary at the cell surface. The prevaling hypothesis is that the Cu(II) would be reduced before by root cell surface ferric reductase (Mukherjee et al. 2006; Welch et al. 1993). Besides, no evidence of a Cu reduction by a plasma membrane reductase in poaceae has been shown yet. The result of this study shows that Cu(I) is found in roots but as Cu speciation was analyzed on a bulk of roots, we cannot assess whether reduction occured at the root surface, in the apoplast or in the symplast. Cu(I) is also translocated to stems and leaves where it represents from 63 to 85 % based on XANES results. In previous XAS study, Cu(I) was detected in *Creosote bush* (Zygophyllaceae) (Polette et al. 2000) and *Thalspi caerulescens* (Brassicaceae) (Mijovilovich et al. 2009), and in small proportions in

Elsholtzia splendens (Laminaceae) (Shi et al. 2008) but our results are the first evidence for the presence of Cu(I) in the tissues of a monocotyledon species.

In treatments Cu100 and Cu100Si, the EXAFS LCF results indicate that Cu was bound to S-containing ligands, in two different types of complexes: Cu(I)S-organic and Cu(I)S-inorganic (Figure IV.13). Copper from Cu(I)S-organic complex can originated from thiol groups present on cysteine or methionine ligand. The considerable content of S-containg ligands to bind Cu may be correlated to the decrease of sulfate content in bamboo leaves, stems and roots (Table IV.3), indeed inorganic S is converted to nutritionally and functionally important S-containing compounds and their synthesis might be a major factor in plant stress-defense (Sirko et al. 2007). Several cysteine-rich binding ligands are involved in the homeostasie, transport and tolerance mechanisms of Cu, namely opper chaperones, Glutathione (GSH), Phytochelatines (PCs) and Metallothioneins (MTs). Copper chaperones are involved in the intracellular trafficking of metal ions and insert the Cu into the active sites of specific proteins (Gonzalez-Guerrero and Arguello 2008). GSH and PCs may be involved in homeostasis and in Cu transport both in the phloem (Mendoza-Cozatl et al. 2008) and xylem (Wei et al. 2007). PCs play an important role in Cu toxicity by forming stable Cu complexes (Clemens 2006; Cobbett and Goldsbrough 2002; Qian et al. 2005). Metallothioneins are also involved in Cu homeostasis and Cu tolerance of *Arabidopsis thaliana* (Guo et al. 2008; Roosens et al. 2004). Therefore the strategy of bamboo to cope with Cu is the internal Cu sequestration by S-rich ligands and probably many of them. The higher proportion of Cu(I)S-compound in aerial part is consistent with the finding of Qian et al. (2005) which shows that PCs have a more active role in the leaves than in the roots.

The presence of a Cu(I)S inorganic ligand is more surprising, and reveal the presence of another Cu S-rich complex. To our knowledge, this is the first time that a Cu(I)S inorganic phase is described. Mineralization could be a defense against toxic copper, but reports of Cu biominerals are rare: copper sulfide mineralization in yeast (Yu et al. 1996), copper oxalate in lichens (Purvis et al. 2008), in fungus (Fomina et al. 2005) and in *Thalspi caerulescens* (Mijovilovich et al. 2009). It has been shown that in plants cadmium may be stored in a stable high-molecular weight complex that contains Cd, PCs and sulfides (Pickering et al. 1999; Reese et al. 1992). Some complexes with high Cd ratios consist of aggregated particles which themselves consist of CdS crystallite core coated with PCs (Bae and Mehra 1998; Dameron and Winge 1990). We could make the hypothesis that a similar process occurs with Cu and PCs and inorganic sulfides, and participate to the storage of Cu in order to reduce available Cu.

Part of Cu in stems and leaves of bamboo is present as a Cu(II)-malate complex. In leaves, we cannot discriminate the Cu(II) as Cu(II)-malate complex from Cu(II)-histidine complex. In plants, S-free ligands include free amino acids (histidine, asparagine, and alanine), organic carboxylic acids (citric, malic etc.) and phytosiderophores (nicotinamine (NA), mugineic acid, avenic acid. Simulation models (Mullins et al. 1986; White et al. 1981a; White et al. 1981b) and in vivo studies of xylem sap (Irtelli et al. 2009; Liao et al. 2000; Pich and Scholz 1996) show that the most important long-distance Cu-chelating compounds are amino acids mostly histidine, asparagine and nicotianamine. In bamboos, the histidine concentration increased significantly in treatment Cu100 and Cu100Si in roots, but not in stems (Table IV.3). Therefore, the role of amino acids could be involved in the Cu complexes, although the concentration of soluble amino acids content is smaller than those of carboxylic acids. For example in stems in treatment Cu100, the average histidine concentration was 0.64 mmol kg^{-1}, whereas malate concentration was 20 mmol kg^{-1} (Table IV.3). Carboxilic acids may also bind to Cu and thus contribute to in its sequestration or transport (Callahan et al. 2006). EXAFS data demonstrated that malonate, citrate appeared unlikely to coordinate Cu(II), while malate was found to be the most likely to do so. Although data resulting from malate or histidine complexes were difficult to separate because of their chemical similarity, their evaluation in LCF and their different concentration in bamboos, somewhat favour malate over histidine as the more likely main ligand.

The speciation of Cu in leaves of Cu1.5 treatment and Cu100 treatment is similar (Figure IV.9), therefore it seems that the bamboo applies the same strategy to cope with Cu concentrations in its tissues that are either toxic or non-toxic.

In leaves and stems, the supplementation of Si in nutrient solution in treatment Cu100Si induced an increase of the Cu(I) proportion compared to treatment Cu100. Recently, Khandekar and Leisner (2011) examined the expression of three MT genes in Arabidopsis, subsequent to a Cu toxicity and Si supplementation which alleviates metal toxicity. Cu induced the expression of these genes, as previously reported (Guo et al. 2003), but the levels of expression either remained elevated or were boosted to even higher levels in plants provided with extra Si. In this study, we can assess that an increase of MTs production in presence of Si may induce an increase in complexes Cu(I)-MT concentration. Therefore, the formation of these complexes will be a strategy to cope with the high Cu toxicity level in cells. However, silicon-mediated alleviation of Cu toxicity has not been confirmed by this study with bamboo *Phyllostachys fastuosa*.

In summary, this study provides important evidences for the presence of Cu(I) bound to sulfur compound in a *Poaceae* species. The main strategy of bamboo to cope with high Cu concentration in its tissues is (i) an important sequestration in the roots apoplast, mainly in epidermis, and (ii) a complexation with Cu(I)S compound organic, that can be phytochelatines, metallothioneins, metalochaperonnes, etc (iii) formation of a Cu(I) sulfur compound inorganic that may be involved in Cu storage. Si supplementation has induced low beneficial effects on bamboo Cu tolerance, but modified the Cu speciation.

5. SUPPORTING INFORMATIONS

SI I References compounds

Table IV.5 Reference compounds database

	references	oxidation state	first shell
Organic compound	Cu(I)-acetate	1	O
	Cu(I)-cysteine	1	S
	Cu(I)-thiophenolate	2	S
	Cu(I)-thioglycolique	2	S
	Cu(II)-histidine	2	N/O
	Cu(II)-malate	2	O
	Cu(II)-malonate	2	O
	Cu(II)-acetate	2	O
	Cu(II)-formate	2	O
inorganic compound	Chalcocite (Cu_2S)	1	S
	Cuprite (Cu_2O)	1	O
	Covellite (CuS)	2	S
	Tenorite (CuO)	2	O
	Dioptase ($CuSiO_2(OH)_2$)	2	O
	Cornetite ($Cu_3(PO_4)(OH)_3$)	2	O
	Malachite ($Cu_2(CO_3)(OH_2)$)	2	O
	Brochantite ($Cu_4(SO_4)(OH)_6$)	2	O
	$Cu(OH)_2$	2	O
	Elemental Cu	0	Cu

An extensive library of Cu model compounds was used and contained 10 inorganic and 9 organic compounds with various Cu oxidation states (Cu(0), Cu(I), and Cu(II)). Inorganic reference compound were described in the study of Legros et al. (2010). The crystalline organo metallic complexes Cu(II)-acetate dihydrate; Cu(II)-formate, Cu(I)-thiophenolate, Cu(I)-acetate were purchased from Sigma aldrich. In addition, 4 crystalline organometallic complexes of known structure were synthesized. The complexes were chosen for their diversity in functional groups (-COOOH, -OH, -SH, $-NH_2$) representative of natural organic matter moieties.

- Copper(II) di(hydrogen malonate) dehydrate (Lenstra and Kataeva, 2001);
- Diaquabis(malato-$\kappa^2O^1O^2$)copper(II) (Zhang, 2007);
- Malonic and malic acid were selected among several hydroxy-carboxylic acids for this database due to their capacity to model copper(II) binding with NOM (Manceau and Matynia, 2010)

- Bis-L-Histidine copper(II) Dinitrate Dihydrate (Evertsson, 1969).
- Copper(I) cysteine (Rigo et al, 2004).

Synthesis of copper(II) complexes with thio-organic acids were attempted: Cu(II) with cystein (Dokken et al., 2001) and Cu(II) with thioglycolic acid (synthesis adapted from Ogawa et al., 1981). Unfortunately, copper reduction was observed resulting in presence of Cu(I). It was thus conducted that Cu(II) is unlikely to be stable in the presence of ligand containing reduced sulfur; these data were not considered in further analysis.

SI II XANES spectra analysis

The minimum number of reference spectra needed to fit the unknown sample was determined by principal component analysis (PCA) (Beauchemin et al. 2002). Principal component analysis was performed using SixPack software (Stanford, CA). The indicator function IND provides a means to determine the number of relevant components and should be minimal for the number of relevant components. For the relevant component, we also verified that their progressive introduction during the spectral reconstruction decreases NSS at least 20 %. Relevant reference compounds were identified by target transformation and the SPOIL function (Malinowski 1978) was used to evaluate if a given model compound was an acceptable target. This is a non-negative dimensionless number for which values <1.5 are considered excellent, 1.5–3 good, 3–4.5 fair, 4.5–6 poor, and >6 unacceptable. The uncertainty in the proportion of Cu species is estimated to be approximatively 10 % of the total amount of Cu (Manceau et al. 2002a).

Table IV.6 gives the PCA output parameters, including the eigenvalue, indicator values (IND) and variability explained by the component (Var) for the nine components. IND is assumed to be minimum when the number of principal components is reached (Manceau et al. 2002b; Sarret et al. 2004a). In this study, IND was minimum with 4 components (0.037). But, the third and fourth components contained essentially noise. Moreover, the whole set of spectra (n = 10) were reconstructed correctly with 2components.

Table IV.6 Component output parameters determined by principal component analysis of the whole set of XANES spectra

Component	Eigenvalue[a]	Var	Cum. Var	IND[b]
1	66.75	0.91	0.91	0.0189
2	4.44	0.06	0.971	0.0064
3	1.05	0.014	0.985	0.0038
4	0.37	0.005	0.99	0.0037
5	0.21	0.002	0.993	0.0044
6	0.18	0.001	0.995	0.0052
7	0.13	0.001	0.997	0.0069
8	0.09	0	0.998	0.0101
9	0.04	0	0.999	0.0377

[a]Eigenvalue : values of the diagonal matrix in the PCA after consecutive elimination of the components. Eigenvalues rank PCs according to their importance to reproduce data.
[b] Malinowski (1978) indicator value.

The nature of Cu species was determined by target transformation and the SPOIL function (Manceau et al. 2002b) was used to assess whether a given model compound was an acceptable target. This is a non-negative dimensionless number for which values < 1.5 are considered excellent, 1.5-3 good, 3-4.5 fair, 4.5-6 poor, and > 6 unacceptable. Table IV.7 shows results of target transform by selecting the two first components.

Table IV.7 **Results of target transform of XANES spectra (Chi square, R value and Spoil values) of the main Cu references**

References	Chi Sq	R value	Spoil
Malachite ($CU_2(CO_3)(OH_2)$)	0.27	0.00059	1.16
Cu(II)-malate	0.28	0.00057	1.33
Cornetite ($Cu_3(PO_4)(OH)_3$)	0.36	0.00081	1.72
Cu(OH)2	0.64	0.00144	2.17
Cu(II)-histidine	0.36	0.00085	2.25
Cu(I)-cysteine	0.51	0.00112	2.4
Chalcocite (Cu_2S)	0.61	0.00134	2.79
Cu(II)-formate	1.32	0.00287	2.9
Cu(I)-thiophenolate	0.59	0.00137	3.07
Cu(II)-malonate	1.75	0.00382	3.16
Cu(II)-acetate	1.09	0.00249	3.24
Cu(I)-thioglycolique	0.76	0.00178	3.25
Brochantite ($Cu_4(SO_4)(OH)_6$)	1.93	0.00429	3.33
Dioptase ($CuSiO_2(OH)_2$)	1.24	0.00274	3.44
Tenorite (CuO)	1	0.00237	5.4
Cu(I)-acetate	1.34	0.00305	6.1
Covellite (CuS)	2.12	0.00502	7.5
Cuprite (Cu_2O)	2.64	0.00597	9.9
Elemental Cu	9.5	0.02629	26.8

Table IV.8 Proportion of Cu species determined by LCF of the Cu K-edge XANES spectra

	treatment	Cu(I)-cysteine	Cu(II)-malate	Cu(II)-acetate	Somme	NSS (%)
leaf	Cu1.5	0.79		0.25	1.04	0.146
	Cu1.5Si	0.72		0.32	1.04	0.144
	Cu100	0.64	0.37		1.01	0.026
	Cu100Si	0.85	0.15		1.00	0.024
stem	Cu100	0.72	0.29		1.02	0.018
	Cu100Si	0.82	0.17		0.99	0.022
root desorbed	Cu100	0.40	0.56		0.95	0.031
	Cu100Si	0.39	0.56		0.95	0.033
root	Cu100	.010	0.84		0.94	0.058
	Cu100Si	0.17	0.78		0.95	0.045

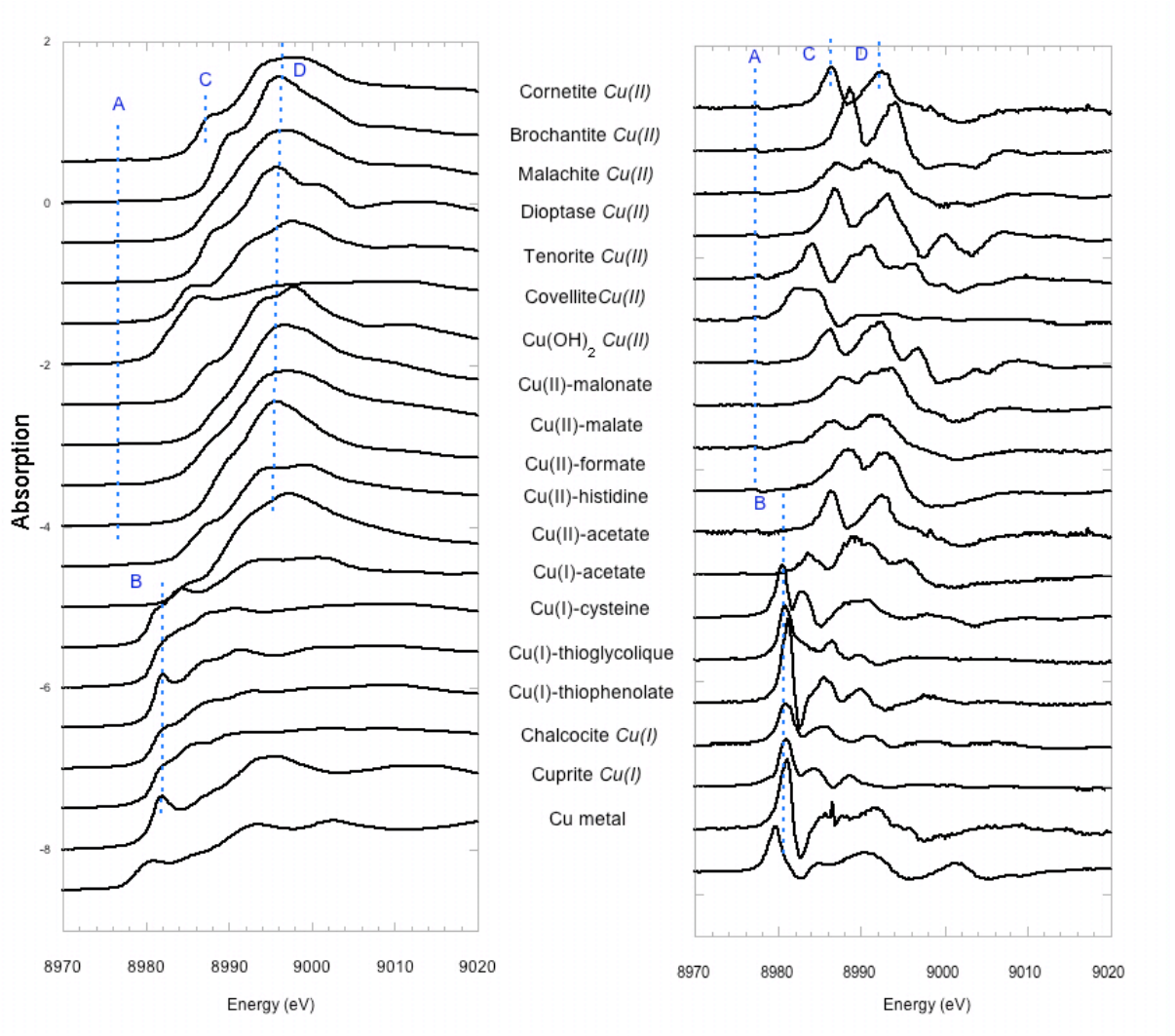
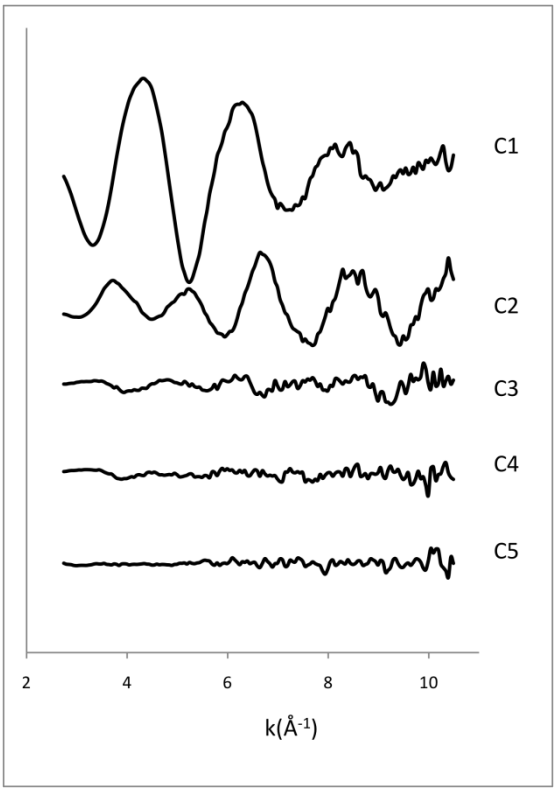


Figure IV.14 XANES spectra and their first derivative of reference compounds

SI III EXAFS spectra analysis

The numbers of complexes identifiable by EXAFS spectroscopy in the samples were evaluated by PCA on the whole set of bamboo spectra (n=8). Based on the IND local minimum criterion, the dataset contains 2 relevant components (Figure IV.15). However, only 66 % of the total experimental variance could be described by the first two components and the examination of the first five principal components suggest that the third component contain signal. It is shown in previous studies that the IND determination may underestimate or overestimated the number of independent component required (Manceau et al. 2002a; Panfili et al. 2005; Sarret et al. 2004b). Moreover, the number of component was estimated here by calculating how much the fit is degraded upon varying the proportions of the reference spectra in the LCF. Using this procedure, the quality of the reconstruction improves significantly with 3 components. Then, the nature of Cu species was determined by target transformation, and the SPOIL function (Malinowski. E.R. 1978; Manceau et al. 2002b) was used to evaluate if a given model compound was an acceptable target (Table IV.9).



Component	Eigenvalue	Var	Cum. Var	IND
1	64.215	0.501	0.501	0.26304
2	31.124	0.243	0.745	0.15803
3	8.873	0.069	0.814	0.19222
4	5.999	0.046	0.861	0.27859
5	5.084	0.039	0.901	0.46974
6	4.692	0.036	0.937	0.99367
7	4.130	0.032	0.970	3.81214
8	3.812	0.029	1.0	NA

Figure IV.15 a) Component spectra of the principal component analysis, b) first to eight component output parameters determined by principal component analysis of a whole set of EXAFS spectra

Table IV.9 Results of target transform of EXAFS spectra (Chi square, R value and Spoil values) of the main Cu references

Reference	Chi Sq	Rvalue	SPOIL
Cu(II)-malate	41	0.03	1.44
Cu(II)-histidine	58	0.06	2.03
Brochantite ($Cu_4(SO_4)(OH)_6$)	215	0.13	2.85
Chalcocite (Cu_2S)	158	0.16	3.00
Cu(I)-cysteine	244	0.15	3.03
Cu(II)-formate	230	0.17	3.39
Cornetite ($Cu_3(PO_4)(OH)_3$)	1172	0.36	3.69
Dioptase ($CuSiO_2(OH)_2$)	373	0.18	4.43
Cu(II)-acetate	246	0.22	4.47
Cu(I)-thiophenolate	193	0.19	4.49
Cu(II)-malonate	531	0.21	4.66
$Cu(OH)_2$	1667	0.49	5.21
Cuprite (Cu_2O)	4911	0.91	6.07
Cu(I)-thioglycolique	731	0.36	6.31
Malachite ($Cu_2(CO_3)(OH_2)$)	386	0.37	6.42
Elemental Cu	29771	0.75	6.82
Tenorite (CuO)	1830	0.57	7.26
Cu(I)-acetate	371	0.71	7.27
Covellite (CuS)	1213	0.46	8.42

Table IV.10 Proportion of Cu species determined by LCF of the Cu K-edge EXAFS spectra

		Cu_2S	Cu(I)-cysteine	Cu(II)-malate	Cu(II)-histidine	somme	NSS (%)
leaf	Cu100	0.22	0.19	0.32		0.73	14.2
	Cu100Si	0.38	0.29	0.21		0.88	12.3
stem	Cu100	0.31	0.26	0.29		0.86	9.8
	Cu100Si	0.31	0.33	0.24		0.88	9.3
root desorbed	Cu100		0.19	0.32	0.33	0.84	4.1
	Cu100Si		0.19	0.54		0.74	6.9
root	Cu100			0.43	0.42	0.85	3.2
	Cu100Si			0.72		0.72	3.9

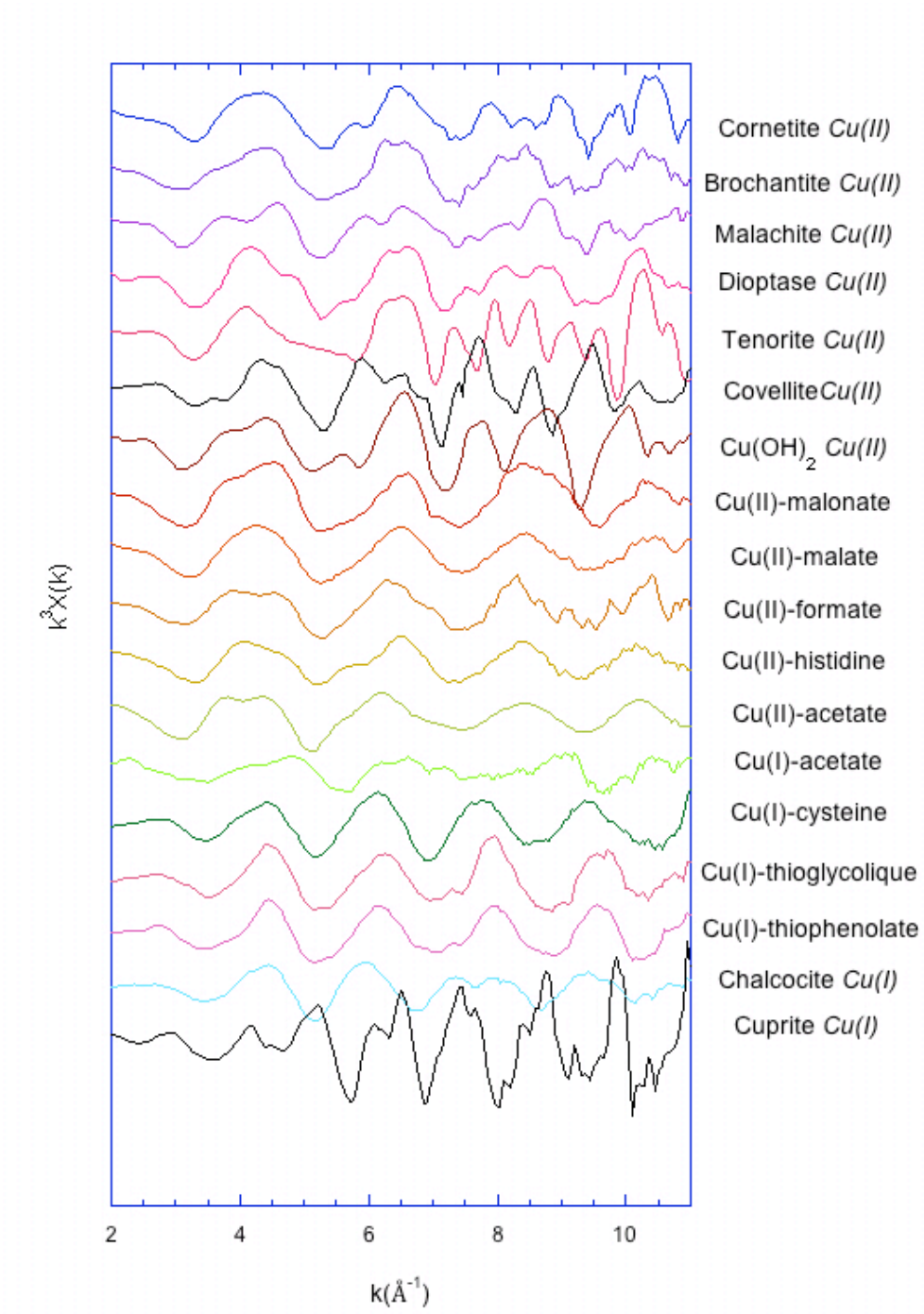


Figure IV.16 EXAFS spectra of reference compounds

6. BILAN DU CHAPITRE IV

Les bambous monopodiaux *Phyllostachys fastuosa* ont toléré une concentration de 1.5 µM Cu^{2+} en solution, en accumulant des concentrations de Cu similaires à celle de l'expérience d'hydroponie précédente effectuée avec des bambous sympodiaux *Gigantocloa sp*. « Malay Dwarf ». En revanche, une concentration de 100 µM Cu^{2+} en solution a induit une forte toxicité chez les bambous : diminution de la biomasse, apparition de chlorose, concentration en Cu dans les tissus élevée, diminution de certains nutriments. L'ajout de silicium n'a pas eu d'effets significatifs sur le développement des bambous quel que soit le traitement, mais a induit une diminution des symptômes visuels de la toxicité de Cu.

Le cuivre est essentiellement accumulé dans les **racines**, ce compartiment retenant 70 à 96 % de Cu du bambou. En raison de sa forte affinité pour les parois racinaires, une part importante de Cu est retenue dans le compartiment apoplasmique et en particulier au niveau des cellules de l'**épiderme**. Cela a été observé par microscopie et est confirmé par la spéciation de Cu racinaire : il est complexé majoritairement sous forme **Cu(II)** par des **composés carboxyliques**, de type malate, ou des **composés aminés** de type histidine. La proportion de ces composés diminue lorsque l'on désorbe le Cu racinaire au profit de composés Cu(I). L'ajout de silicium à entraîné une augmentation de la proportion de Cu apoplasmique et modifié la spéciation de Cu (forme Cu(II)-malate uniquement), sans toutefois entraîner la formation de liaison Si-Cu.

Le cuivre présente **deux états d'oxydation** dans les tissus du bambou : Cu(I) et Cu(II), et ce dès la racine. Les plantes exposées à 1.5 µM et 100 µM Cu^{2+} semblent avoir des stratégies similaires pour faire face à la toxicité du Cu dans la plante. Dans les parties aériennes, Cu(I) est majoritairement lié à des **composés organo-sulfurés** de type cystéine, mais aussi à des **composés inorganiques soufrés**. Une proportion mineure de Cu est présente sous forme oxydée Cu(II) liée à des **composés organiques non soufrés** : des acides carboxyliques ou aminés de type malate ou histidine. L'ajout de silicium augmente sensiblement la proportion de composés Cu(I) soufrés dans les bambous exposés à 100 µM Cu^{2+}. Le silicium, en induisant l'augmentation de la proportion de Cu complexée à des ligands organo-sulfurés, pourrait alors diminuer la toxicité de Cu.

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CONCLUSIONS ET PERSPECTIVES

Rôle bénéfique du silicium chez le bambou

Ces travaux de recherche trouvent leur origine dans le postulat suivant : l'apport de Si améliore la croissance et la tolérance des bambous aux métaux, permettant une amélioration de la technologie de phytoremédiation. On assiste, depuis une vingtaine d'années, à une augmentation du nombre d'études concernant le Si dans les plantes. Certaines abordent la question de l'essentialité, d'autres de l'identification des transporteurs, d'autres encore de son rôle face à de nombreux stress biotiques ou abiotiques. Si compréhension du transport de Si dans les plantes a progressé, on remarque toutefois que les mécanismes d'action du Si permettant à la plante de réduire l'impact d'un stress sont très divers et qu'il convient de les étudier. L'action du silicium semble être différente selon les types de stress, les espèces étudiées, les conditions environnementales, etc.

Les bambous possèdent une forte capacité à accumuler Si, principalement dans les feuilles. Ce travail a permis de montrer que cette capacité varie significativement entre les espèces, et particulièrement entre les deux principaux types de bambous : les bambous monopodiaux, à rhizomes traçants et les bambous sympodiaux, à rhizomes cespiteux. Grâce aux expérimentations en hydroponie, nous avons mesuré une plus faible concentration de Si dans les racines que dans les tiges et les feuilles. Ce résultat, qui est en désaccord avec les concentrations de Si mesurées dans les racines de bambous lors d'études publiées précédemment (Ding et al. 2008; Lux et al. 2003), est néanmoins important pour comprendre le mécanisme d'accumulation de Si dans le bambou. La répartition de Si dans les bambous, et le suivi de sa concentration dans les solutions nutritives (notamment un appauvrissement total de Si en solution) confirme la présence de mécanismes d'absorption à la fois actifs et passifs.

Au cours des expériences d'hydroponie, Si n'a pas eu d'effet bénéfique sur le développement des bambous lorsqu'ils ne sont pas soumis à un stress et ce, malgré leur forte capacité à accumuler Si, que ce soit dans le cas d'un bambou sympodial *Gigantocloa sp* « Malay Dwarf » ou d'un bambou monopodial : *Phyllostachys fastuosa*. Face aux deux concentrations de Cu testées dans les cultures hydroponiques (1.5 µM et 100 µM), l'apport de Si ne modifie ni les macronutriments

absorbés, ni les teneurs en acides inorganiques, acides organiques ou acides aminés. Cependant, face à une forte toxicité de Cu (100 µM), la présence de Si dans le milieu induit une légère diminution des symptômes visuels de la toxicité de Cu ainsi qu'une modification de la spéciation de Cu au niveau des racines et des parties aériennes. L'apport de Si pourrait donc diminuer la toxicité de Cu par une plus forte immobilisation de Cu dans l'apoplasme racinaire, et une augmentation dans les parties aériennes de ligands complexants tels que les métallotionéines ou les phytochélatines.

Variabilité du cuivre dans les différentes espèces de bambou

Ce travail est le premier à étudier le cuivre dans le bambou : à savoir les concentrations absorbées en conditions naturelles d'une part et sa toxicité d'autre part. Dans les conditions naturelles pédoclimatiques de l'île de la Réunion, les concentrations en Cu du bambou sont faibles mais similaires à celles de la canne à sucre, une autre Poacée (*Poaceae)* développée sur des sols semblables (Collin and Doelsch 2010). Malgré cela, les concentrations mesurées entre les différentes espèces sont significativement différentes, et il apparaît que les bambous sympodiaux accumulent plus de Cu que les bambous monopodiaux. Nous avons alors testé, au cours de nos expériences en hydroponie, la réponse à un apport de Cu dans deux espèces de bambous appartenant aux deux différents types. Les bambous sympodiaux *Gigantocloa sp* « Malay Dwarf » comme les bambous monopodiaux *Phyllostachys fastuosa* ont présenté la même réponse face à la concentration de Cu appliquée en solution (1,5 µM Cu^{2+}) : concentration de Cu similaire dans les différents tissus, absence de symptômes de toxicité. Ainsi, nous n'avons pas mis en évidence de différence de réaction entre les deux types de bambous lorsqu'ils sont exposés à une forte concentration de Cu contrairement à ce qui avait été observé dans les conditions environnementales des sols de la Réunion.

Stratégie de tolérance au cuivre chez le bambou

La première et principale stratégie du bambou pour tolérer Cu est la séquestration de Cu dans ses racines, diminuant ainsi la quantité de Cu internalisée et potentiellement toxique. Cela s'explique aisément par sa forte affinité aux parois cellulaires. Une grande partie de Cu racinaire est en effet présente dans le compartiment apoplastique et localisée, en particulier, au niveau des cellules épidermiques des racines. Ce mécanisme semble être suffisant pour tolérer une concentration de 1,5 µM Cu^{2+} en solution. En effet, aucun signe de toxicité n'a été détecté chez les bambous exposés à cette concentration, que ce soit sur les racines ou sur les parties aériennes.

La seconde stratégie pour gérer Cu est une complexation *in planta* qui permet de diminuer sa toxicité. Malgré les nombreux travaux dédiés à Cu dans les plantes, sa spéciation au sein des

tissus est peu connue. Un résultat important apporté par cette étude est la mise en évidence de Cu réduit dans les racines. Cela avait été observé chez deux dicotylédones (Mijovilovich et al. 2009; Polette et al. 2000), mais cette étude apporte la première évidence de la présence de Cu(I) dans une *Poaceae*. Comme Cu est un cofacteur de nombreuses enzymes, la présence de Cu(I) et de Cu(II) dans la plante est en accord avec les fonctions physiologiques qui lui sont conférées. Une forme minoritaire de Cu est présente dans les parties aériennes sous forme de Cu(II)O/N-organique, forme qui peut être impliquée dans le transport, en formant des complexes à des acides carboxyliques ou acides aminés. Nous montrons dans cette étude que la phase majoritaire de Cu dans les parties aériennes du bambou est sous forme de Cu(I) complexé à des composés soufrés de deux types : un composé Cu(I)S-organique et Cu(I)S-inorganique. Cette étude est la première à détecter la présence d'un composé soufré inorganique dans la plante. Un complexe de type Cu(I)S-organique indique une complexation à des acides aminés soufrés tels que la cystéine et la méthionine, qui sont des constituants de nombreuses molécules impliquées dans l'homéostasie, le transport et le stockage.

PERSPECTIVES

Spéciation de Cu dans la plante

Dans les tiges et les feuilles, nous avons identifié un complexe Cu(I) inorganique soufré. Il serait intéressant de confirmer la présence de cette phase. Plusieurs techniques sont utilisées pour identifier la localisation des éléments traces dans les plantes, comme par exemple la spectroscopie par perte d'énergie d'électrons (EELS) (Liu and Kottke 2004), l'analyse par faisceaux d'ions (proton-induced X-ray émission : PIXE)(Kramer et al. 1997), la micro-fluorescence X induite par rayonnement synchrotron (Sarret et al. 2009; Shi et al. 2011). Ces techniques sont le plus souvent utilisées pour une étude à l'échelle du tissu ou des cellules, avec une résolution spatiale de l'ordre de 1 µm. Le développement récent de l'utilisation du NanoSIMS (Nano-scale Secondary Ion Mass spectrometry) en biologie semble particulièrement intéressant. En effet, cette technique permet d'analyser des échantillons avec une très bonne résolution spatiale (<50nm), une très bonne sensibilité, tout en discriminant clairement tous les éléments et isotopes présents (Smart et al. 2010). Des études récentes ont utilisé cette technique pour étudier la distribution du nickel dans les cellules d'une plante accumulatrice (Smart et al. 2010), localiser l'arsenic et le sélénium dans des graines de céréales (Moore et al. 2010), ou la distribution subcellulaire du silicium et de l'arsenic (As) dans les racines de riz (Moore et al. 2011). Dans cette dernière étude, les auteurs sont capables de localiser le silicium sur les parois

cellulaires de l'endoderme et de co-localiser l'arsenic et le soufre dans les vacuoles de certaines cellules, confirmant une forme de stockage de type As-phytochelatin (Moore et al. 2011). Les analyses nanoSIMS s'effectuant sur des échantillons secs, la contrainte majeure de l'utilisation du nanoSIMS est la préparation des échantillons qui doit préserver les structures morphologiques et conserver la distribution chimique (Grovenor et al. 2006).

Ce travail de thèse a apporté des résultats importants concernant la spéciation de Cu et la localisation de Cu et Si dans la racine. Mais l'utilisation d'une technique d'imagerie ayant une meilleure résolution spatiale et une meilleure sensibilité pourrait être utilisée pour localiser les éléments dans les cellules des tiges et des feuilles. Cela pourrait permettre de mieux comprendre le comportement de Cu dans la plante, comme par exemple la localisation des complexes Cu-S dans les cellules : sont-ils séquestré dans la vacuole, dans le cytoplasme ? Les complexes Cu-O/N organiques sont-ils des formes de transport, ou bien des formes de stockage ? Au niveau racinaire, cela permettrait également de mieux comprendre le rôle du silicium observé lors de la seconde expérience d'hydroponie. Nous avons vu que la présence de Si augmente la proportion de Cu apoplasmique et modifie sa spéciation. Il est possible que des observations comme celles qui sont décrites dans l'étude de Moore et al (2011) pourraient révéler des distributions différentes des éléments en présence de Si, et donc une meilleure compréhension de son rôle dans la racine.

La famille des Poacées, dont fait partie le bambou, représente plus de 20 % de la couverture végétale terrestre et rassemble de nombreuses espèces essentielles pour l'alimentation humaine (Arber 2010). Il est probable que le comportement de Cu présente des similarités entre les espèces de Poacées. Ainsi, une meilleure connaissance de la spéciation de Cu représente une avancée importante pour estimer sa toxicité au cours de la chaîne alimentaire, son devenir dans les sols, etc.

Il serait donc intéressant, pour poursuivre ce travail, d'étudier la spéciation et la localisation de Cu dans d'autres Poacées. De plus, même si le bambou à un fort taux de croissance, son développement en hydroponie est long et sa réponse face à une forte toxicité n'est observable qu'après un mois de culture. Afin d'avoir des résultats quantifiables, nous avons dû mener nos deux expériences de 11 mois et 6 mois respectivement, en incluant le temps de pré-culture. Ainsi, l'utilisation d'une Poacée plus facile à cultiver (croissance à partir de graines, développement plus rapide) telle que le blé, serait intéressante pour compléter les résultats de spéciation et de localisation de Cu. En outre, nos résultats nous renseignent sur l'effet d'une toxicité à long terme de Cu chez le bambou, ce qui est adapté au contexte de phytoremédiation. Cependant comme les métaux induisent une toxicité dès les premières heures d'exposition de la

plante (ex, Blamey et al. 2011; Kopittke et al. 2011) il serait également intéressant d'effectuer des études à court terme afin de comprendre la réponse des plantes à une toxicité de Cu.

Amélioration de la technologie de phytoremédiation

Le cuivre est un contaminant abondant des effluents qui peuvent être traités par le BAMBOU ASSAINNISSEMENT® ; la compréhension de son comportement, des mécanismes de prélèvement et de toxicité, est un enjeu fort pour améliorer la technologie.

Il est délicat d'extrapoler les résultats obtenus en culture hydroponique à ceux que l'on pourrait obtenir sur un sol en conditions naturelles. Toutefois, il semble que le bambou soit capable de tolérer de fortes concentrations de Cu. Nous avons vu que la forte capacité à tolérer Cu est liée à sa séquestration dans les racines, et notamment à sa forte adsorption sur les parois cellulaires. Ainsi, une augmentation de biomasse augmente la quantité de sites d'adsorption disponibles. Il semble donc que, grâce à son fort taux de croissance, le bambou présente des capacités de tolérance à Cu supérieures à d'autres espèces, le blé par exemple (Bravin et al. 2010). Dans un sol, la forte production de biomasse racinaire du bambou pourrait permettre des mécanismes similaires d'adsorption de Cu sur les parois cellulaires des racines, et ainsi une bonne tolérance à Cu.

Cette tolérance à Cu permet au bambou de conserver son efficacité pour l'épuration des autres polluants. La majeure partie de Cu prélevée par le bambou est difficilement exportable de la parcelle puisque elle est localisée dans les racines. En revanche, cette capacité à séquestrer Cu dans ses tissus racinaires semble être intéressante pour une utilisation du bambou dans un objectif de phytostabilisation. Cela permettrait de réduire la mobilité de Cu dans le sol en l'immobilisant *in situ*, et de réduire ainsi les risques de dispersion dans le milieu environnant.

Nous avons montré que le silicium n'influence pas directement la croissance et le développement du bambou en conditions contrôlées. Cependant, en conditions environnementales, les bambous sont exposés à de nombreux stress non étudiés ici (des stress salins, stress hydriques, stress biotiques…), par conséquent Si pourrait avoir un rôle indirect mais bénéfique sur le bambou. Enfin, il n'est pas exclu non plus que Si puisse avoir un rôle plus important dans le bambou face à une toxicité induite par d'autres métaux, comme le cadmium par exemple, ou face à une pollution multi métallique comme cela a été décrit récemment pour le riz (Gu et al. 2011).

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RESUME

Cette étude vise à évaluer le rôle du silicium (Si) sur l'amélioration de la croissance et de la tolérance au cuivre (Cu) du bambou, plante utilisée en phytoremédiation. Plusieurs approches ont été choisies. Dans un premier temps, la répartition et la variabilité de Cu et Si ont été étudiées dans plusieurs espèces de bambous se développant dans un contexte pédoclimatique naturel afin d'établir des concentrations de référence pour ces éléments. Des cultures hydroponiques ont ensuite permis de caractériser de manière macroscopique la réponse des bambous à des apports de Si et Cu, et, parallèlement, d'étudier la spéciation de Cu et la localisation de Si et Cu au sein des différents organes du bambou (racines, tiges, feuilles).

Les concentrations en Si et Cu présentent des différences significatives entre les principaux types de bambous, suggérant l'importance d'un caractère génotypique responsable de l'absorption de ces éléments. Face à une toxicité au cuivre, un apport de Si modifie la spéciation de Cu dans les tissus du bambou, sans toutefois améliorer significativement sa tolérance. Il apparaît que la stratégie principale du bambou pour gérer de fortes concentrations de Cu est tout d'abord une importante séquestration dans les racines, puis une complexation de Cu avec des composés soufrés organiques et inorganiques. Les résultats de cette étude permettront d'optimiser les technologies liées aux capacités épuratives des bambous face à une pollution métallique.

Mots clefs : silicium, cuivre, bambou, phytoremédiation, culture hydroponique, spectroscopie d'absorption X (EXAFS, XANES)

ABSTRACT

This study aims at assessing the role of silicon (Si) on the plant growth and alleviation of copper (Cu) toxicity in bamboos. Several approaches have been performed. Firstly, the distribution and variability of Si and Cu were investigated in several bamboo species grown under natural pedo-climatic conditions in order to obtain reference values for Cu and Si in bamboos. Secondly, hydroponic experiments were carried out to characterize the macroscopic response of bamboo plants exposed to Si and Cu and, investigated in parallel the Cu speciation and Si and Cu localisation in different part of bamboos (roots, stems, leaves).

Significant differences were measured between bamboo species, suggesting that a genotypic character may be responsible for Si and Cu accumulation. Silicon supplementation modified the Cu speciation but did not induce significant improvement of Cu tolerance. The main strategy of bamboo to cope with high Cu concentrations in its tissues is initially an important sequestration in the roots apoplast, mainly in epidermis, and then a Cu complexation with organic and inorganic sulphur compounds. These results will allow the optimisation of phytoremediation processes using bamboo plants.

Keywords: silicon, copper, bamboo, phytoremediation, hydroponic, X-ray absorption spectroscopy (EXAFS, XANES)